Structural Bioinformatic Approaches to the Discovery of New Antimycobacterial Drugs

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Abstract: Integrated bioinformatic approaches to drug discovery exploit computational techniques to examine the flow of information from genome to structure to function. Informatics is being be used to accelerate and rationalize the process of antimycobacterial drug discovery and design, with the immediate goals to identify viable drug targets and produce a set of critically evaluated protein target models and corresponding set of probable lead compounds. Bioinformatic approaches are being successfully used for selection and prioritization of putative mycobacterial drug target genes; computational modelling and x-ray structure validation of protein targets with drug lead compounds; simulated docking and virtual screening of potential lead compounds; and lead validation and optimization using structure-activity and structure-function relationships. Active sites can be identified, characterizing patterns of conserved residues and, where relevant, predicting catalytic residues, thus providing information to aid the design of selective and efficacious pharmacophores. In this review, we describe selected recent progress in antimycobacterial drug design, illustrating the strengths and limitations of current structural bioinformatic approaches as tools in the fight against tuberculosis.

Key Words: Tuberculosis, structural bioinformatics, mycobacteria, virtual screening, structure-based drug design.

INTRODUCTION

The Role of Bioinformatics in Drug Discovery

Let us define bioinformatics in a most general sense as a complex landscape of computational methods that utilize data base mining, machine learning techniques, and predictive modeling to provide discovery of information or knowledge useful to our goal of developing new antimycobacterial drugs. We can then recognize several synergistically interacting areas, in which bioinformatics contributes increasingly and dominantly to our goal:

- *Sequence-derived information from genomic databases*. Accurate multiple sequence alignment enables comparative modeling, prediction of functional residues, secondary structure prediction, phylogenetic methods and deduction of characteristic sequence motifs or protein families. Sequence-derived information provides rapid initial annotation of genomes, accelerates target selection by discrimination of specific *Mycobacterium tuberculosis* genes from host genes, or allows reconstruction of putative metabolic pathways comprised of novel and attractive drug targets.
- *Detailed three-dimensional structure information from crystallographic, nuclear magnetic resonance or homology modeling methods.* Structural bioinformatics exploits three-dimensional, highly detailed structural information to provide a basis for the design of novel drugs, which are largely inhibitors of a critical function in a unique pathway. Predictive modeling of structures using experimentally determined structures of homologous templates (> 30% sequence identity) has been shown to provide accurate models (>80%; rmsd < 3.5Å), which can be used to support site-directed mutagenesis, to design chimeras and crystallizable variants, for molecular replacement in crystallography, to define antibody epitopes, for virtual screening and docking of small ligands, to dock macromolecules and predict protein partners, to design and improve ligands, and to study catalytic mechanism [1].
- *In silico screening of molecular libraries.* Theoretical prediction of the association of flexible ligands, such as "drug-size" molecules [2], with protein targets, is a powerful tool for understanding molecular function and mechanism, as well as predicting binding affinity [1]. A variety of available computational methods are capable of locating binding conformations that closely approximate experimentally determined structures [36]. Although candidate drug leads of inhibitory potential may be identified through structure-guided approaches, their "drugability" must also be assessed using the established first-line approximation known as Lipinski's "rule of 5" [2, 3]. Advanced computational tools are capable of predicting ADME/T properties (absorption, distribution, metabolism, excretion, toxicity), and exploring a much more extensive array of possible interactions that affect solubility, stability, bioavailability, and membrane permeability [4-6].

In this review, we will focus on the central role of structure-based drug design methods, with extensions, where appropriate, to the equally important front and back end

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analysis of genomes and the potential of drug leads. With the advance of high throughout crystallography screening techniques in the structural genomics initiatives [7], structure-based drug design is now moving towards the realm of true structure-based drug discovery [8]. The number of detailed examples will necessarily be limited, but these case studies have been selected to highlight recent advances and successes, and to provide an overview broad enough to appreciate both the power and limitations of current informatics-based approaches. The aim of this presentation is thus to guide the experimental or clinical researcher in deciding when informatic approaches, in particular structural bioinformatics, can be valuable tools in the fight against tuberculosis.

The Global Threat of Multidrug Resistant TB

In 1993, the World Health Organization (WHO) declared tuberculosis (TB) as a "global emergency" [9]. More than two million people die each year of the disease [10], and it is expected that there will be 10.2 million new cases by 2005 if the present trend continues. Unfortunately, drug-resistant mycobacteria are "running amok", and multidrug resistance against at least two first-line antimycobacterial therapeutics (MDR) is now the norm [11]. MDRTB has emerged as a major public health threat, because the main response is treatment of infected individuals with medication appropriate to the resistance pattern of their organism, and the supply of second line drugs is limited. Drug-resistant strains are costly to treat, typically hundreds of times more expensive than for sensitive strains. In poor countries, where drugs are either not available or very expensive, treatment of MDRTB is a challenge.

Of even greater concern is the virtual absence of new TB drug development, because most major pharmaceutical companies do not see an adequate return on investment. The first line drugs, isoniazid, rifampicin, pyrazinamide and ethambutol, have increased in price by an average of 11% each year over the past 20 years, due to market failure, monopoly position or patent protection [12]. In addition, the second line drugs needed in the treatment of MDRTB are often considerably toxic. A further complication arises from the extent to which people travel globally, often carrying active drug-resistant strains and potentially spreading the disease. On the basis of currently available data, it has been recommended that a three part response be orchestrated against MDRTB: 1) widespread implementation of short-course chemotherapy as the cornerstone of control; 2) improved resistance testing and surveillance; 3) careful introduction of second-line drugs after evaluating their cost, effectiveness and feasibility [13].

Exploiting Unique Features of *Mycobacterium tuberculosis*

The *Mycobacterium tuberculosis* complex comprises pathogens characterized biologically by slow growth rates (generation time 24 hours), dormancy within the host, a complex cell envelope, intracellular pathogenesis, and genetic homogeneity. An immense body of information about the protein content of the proteome of *M. tuberculosis* became available with the completion of the genome sequence of the H37Rv strain in 1998 [14].Genomic analysis highlighted the importance of lipid metabolism in the life of the tubercle bacillus, the presence of novel biosynthetic

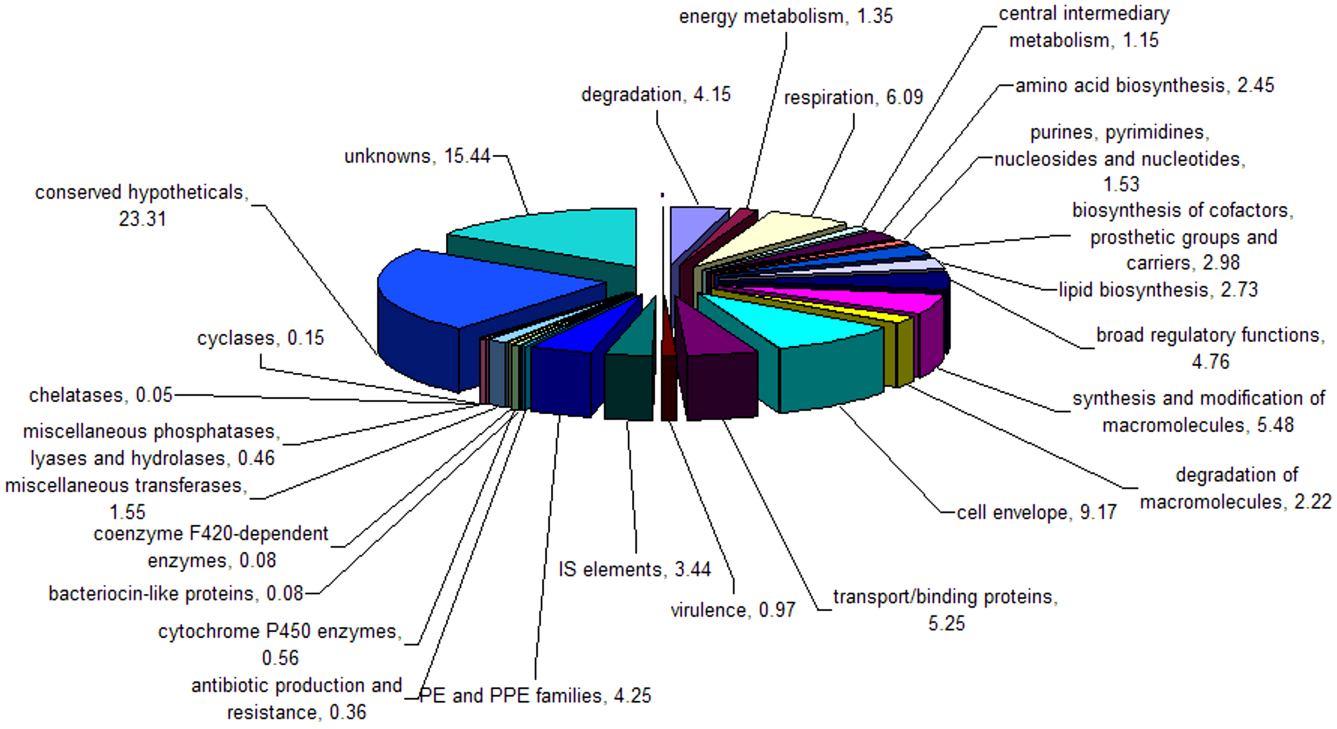

Fig. (1). *M. tuberculosis H37Rv genome composition*. Percentages of genes encoding different functional classes of proteins, based on annotation in [51]. The genome encodes proteins in a number of novel biosynthetic pathways generating cell wall components, and the existence of novel protein families, such as PE and PPE, unique to mycobacteria, which are all attractive drug targets.

pathways generating cell wall components, and the existence of novel protein families, such as PE and PPE, unique to mycobacteria (Fig. **1**) [15]. *M. tuberculosis* has relatively few environmental signal transduction pathways, but the genome encodes a number of proteins similar to eukaryotic serine/threonine kinases, which are believed to be involved in controlling cell dormancy and cell division. The tuberculosis complex is resistant to many antibiotics and includes multidrug resistant forms. This resistance is due largely to the thick, extremely hydrophobic cell envelope of the bacterium, which acts as a barrier to permeability, as well as other resistance determinants encoded by the genome, including many involved in lipid metabolism. The most well-known of these is the sole enoyl-ACP reductase in *M. tuberculosis*, InhA [16], which catalyzes the NADH-dependent reduction of the trans double bond between the C2 and C3 positions on fatty acyl substrates of length C16 or greater. InhA is inhibited by one of the first line drugs in the treatment of TB, isoniazid. Isoniazid is actually a pro-drug, which is activated through the addition of the nicotinamide ring of NADH by the enzyme KatG, a catalase-peroxidase [17]. It is this adduct that is the potent inhibitor of InhA. High level resistance to isoniazid, together with rifampicin [18], another first line drug, constitutes classical MDRTB.

Target Selection and Structural Bioinformatics

The process of drug discovery in prokaryotes has been thought of as successful for more than fifty years, but in fact, the number of bacterial targets actually utilized is less than 0.1% of the potential targets, given the number of bacterial genes identified by sequencing projects [19]. In the past 30 years, only one fundamentally new class of antibiotics has been introduced, the oxazolidinones [20], underscoring the need for new strategies to discover novel antimicrobials. Disease association alone does not create a protein target, and having a good biological rationale does not guarantee that a protein is tractable to chemistry ("drugable"). Biochemical function and drugability are defined by 3D structure, not strictly sequence, because homology implies common evolutionary origin, and 3D structure is more highly conserved than sequence. Rational selection of highly specific gene targets, accompanied by computational protein target modeling [21, 22], virtual screening of small molecule ligands [21, 23], and simulated docking [24-26] are powerful integrated bioinformatic approaches to rapidly identify viable drug targets and drug leads, with initial validation through structure determination. Reliable protein models can also be used to solve experimental x-ray crystal structures by molecular replacement [27]. These methods can accelerate and rationalize the process of antimycobacterial drug discovery, reducing failures early in drug development rather than filling the pipeline with poorly chosen products that have an unknown potential and may ultimately fail at a high cost.

We have entered a post-genomic, post-antibiotic era, where new targets for the discovery of antimicrobials are needed. Results from the sequencing of the genome of the *M. tuberculosis* laboratory strain H37Rv [28] and the clinical strain CDC 1551 [29], as well as several related mycobacteria [30, 31] offer an exceptional opportunity to attack the problem of MDRTB using bioinformatics-based approaches. Instead of first moving to the laboratory, we utilize a variety of sequence, structure and biochemical databases, analyzing and assessing the available data. Comparative genomic techniques can identify putative new antimycobacterial drug targets, such as those that are essential and/or restricted to the mycobacterial system, or genes encoding proteins with unknown functions, such as PE or PPE [32]. Non-traditional targets may also include genes involved in general virulence, disease pathogenesis, adaptation and growth within the host, or novel pathways, such as enzymes involved in lipid metabolism or cell wall biosynthesis. Identification of genes under positive selection or adaptive evolution has also been shown to be a reliable method for identifying novel virulence-associated genes and promising leads for drug targets [33].

The three-dimensional structure of a drug target, experimentally determined or theoretically modeled, particularly if it is complexed with a putative ligand, is required to gain understanding of the mechanism of action of a drug at the molecular level, to appreciate the structural consequences of genetic variations, and to build accurate pharmacophores for drug design. The Protein Data bank contains over 20,000 structures [34], and the emergence of high-throughput structure determination efforts [7] promises the availability of more three-dimensional structures that will be of pharmacogenomic interest. Bioinformatic approaches to drug discovery rely heavily on structural analyses that incorporate homology modeling [35], protein-ligand docking [24, 36], protein-ligand inverse docking [37] and protein-protein docking [38, 39] methods.

A variety of advanced homology modeling methods have been developed, which can provide reliable models of proteins that share 30% or more sequence identity with a known structure [35]. Many of these methods are fully automated on web-based servers, while others require a more knowledge-based approach and are resident on the user's computer. Homology-derived models may be used to identify the location and functional significance of residues implicated in genetic variation or drug action, and "drug-size" molecules [2] may be docked into binding pockets of proteins in order to predict affinity. Methods based on multiple conformer shape matching, genetic algorithms, evolutionary programming, simulated annealing, fragment-based docking and other novel approaches have been shown to be capable of finding ligands with binding conformations at receptor sites close to experimentally determined structures [36]. Inverse docking [40] exploits this binding knowledge to identify multiple protein targets to which a particular small molecule can bind, facilitating prediction of protein targets not only with 'good' biological activity, but also perhaps those implicated in toxicity and side effects of a drug or drug candidate. Finally, we must also consider proteins that interact with other proteins as activators, inhibitors and modifiers. Recent progress in protein-protein docking algorithms [41] has produced a number of models that closely predict experimentally determined structures of complexes, and provide options for increasing our understanding of how two protein surfaces interact. This has implications for the design of antibodies, as well as peptides that block essential protein interactions.

CASE STUDIES: STRUCTURE-GUIDED ANTIMYCOBACTERIAL DRUG DESIGN

Current Drugs that Inhibit Cell Wall Biosynthesis

The mycobacterial envelope consists of a cytoplasmic membrane and cell wall components, peptidoglycan and arabinogalactan, connected to each other in a complex network (Fig. **2**) [42]. Because the cell wall of *Mycobacterium spp*. is required for growth and survival [43], the proteins responsible for the biosynthesis of its components and its formation have become the intense focus of drug discovery efforts [44, 45]. The core of the mycobacterial cell wall is composed of a covalently linked complex of mycolic acids, arabinan, and galactan, attached to peptidoglycan by a Rhap(1-> 3)GlcNAc-P linker unit [44]. The lipid bilayer is asymmetric and dynamic, in which the mycolic acid monolayer, interspersed with porin-like proteins, is perpendicular to the arabinogalactan-peptidogloycan complex, and is complemented by a variety of phospholipids and glycolipids, which together render the lipid barrier relatively impermeable [46]. Because cell wall components produced by these putative protein targets are unique to the pathogen and foreign to the host, elucidating the structure and mechanism of the biosynthetic apparatus of the mycobacterial cell wall should enhance current drug discovery efforts. Indeed, many of the current first-line antitubercular drugs and chemotherapeutics in development, target proteins involved in building and maintaining the mycobacterial cell wall [44, 45].

ISONIAZID

Owing to the essential role of mycolic acids in the mycobacterial cell wall [42, 47], inhibitors of enzymes involved in the biosynthetic pathway of mycolates should have potential antimycobacterial activity. Isoniazid (INH, isonicotinic acid hydrazide) is the oldest synthetic antitubercular which, along with several derivatives, was first reported to be effective in the treatment of tuberculosis 50 years ago [48, 49]. Resistant strains of *M. tuberculosis* were reported shortly thereafter, and it was noted that there was a correlation between resistance and attenuated activity by a catalase-peroxidase [50], since found to be encoded by the *katG* gene (Rv1908c) [17]. Subsequent studies have revealed the structural mechanism of drug resistance, whereby INH is converted by the *katG* encoded catalase-peroxidase into a number of electrophilic intermediates capable of oxidizing or acylating amino acid residues [51]. Using a genetic approach to isolate the isoniazid protein target, a single open reading frame was identified, the *inhA* gene (Rv1484), encoding a NADH-dependent enoyl-ACP

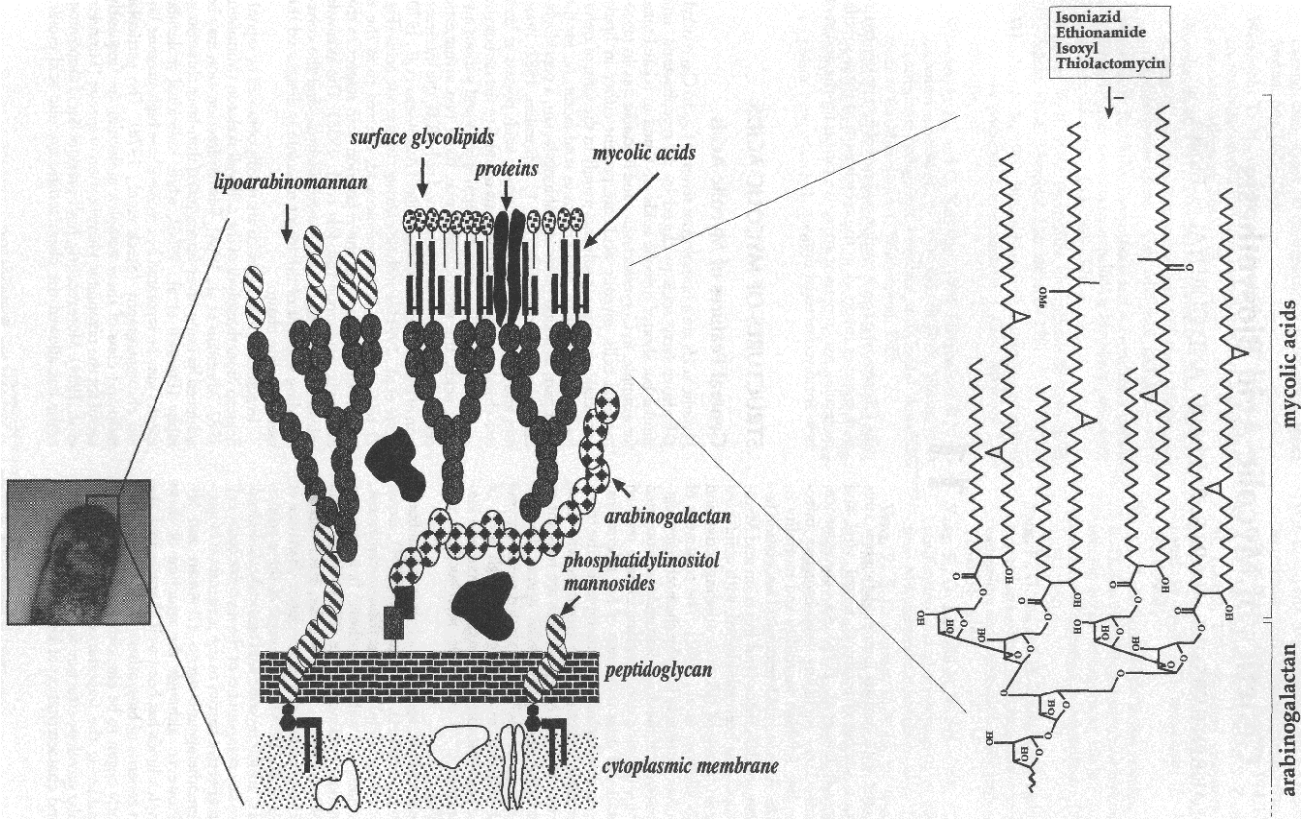

Fig. (2). *Model of the M. tuberculosis cell envelope*. The envelope consists of cytoplasmic membrane and cell wall components peptidoglycan, arabinogalactan, and mycolic acids connected to each other. Lipoarabinomannan and the phosphotidylinositides are shown anchored to the plasma membrane, and glycolipids are presented located to the outside of the envelope *via* interactions with the cell wall. The inset depicts the structure of the terminal tetramycolyl-hexaarabinoside unit of arabinogalactan. Reproduced with permission from [42]. Because the cell wall of *Mycobacterium spp*. is required for growth and survival [43], the proteins responsible for the biosynthesis of its components and its formation have become the intense focus of drug discovery efforts [44, 45].

(acyl carrier protein) [16]. The activated drug binds to and inhibits reductase activity in the presence of NADH or NAD+ [52].

Although mutations within the *inhA* gene facilitate isoniazid resistance [16, 53], InhA remains a good target for structure-guided drug design. The majority of mutations in clinically resistant *M. tuberculosis* isolates are associated with KatG. Furthermore, the *M. tuberculosis* genome encodes for only one enoyl-ACP reductase, InhA, which has longer chain fatty acyl substrate specificity than enoyl-ACP reductases from other sources. InhA belongs to the FAS-II biosynthetic pathway [54, 55], which is absent in humans and superceded by a FAS-I system that is insensitive to many FAS-II inhibitors. Moreover, microbial pathogens appear unable to survive solely by scavenging host fatty acids.

In this regard, *M. tuberculosis* is unconventional among pathogens that invade the human respiratory tract, because it establishes a latent state [56], during which its long-term survival depends on a biochemical pathway known as the glyoxalate shunt, whose enzymes are activated during the metabolic attenuation that accompanies oxygen withdrawal [57, 58]. Structure-guided approaches targeting an essential enzyme in this pathway, isocitrate lyase, are discussed in the section on targeting persistence.

Full characterization of the active site of InhA is now available to facilitate the drug design process from crystal structures of *M. tuberculosis* InhA [59-62] and its homologs from *E. coli* (FabI, formerly EnvM) [63], *Brassus napus* (ENR) [64], and *Plasmodium falciparum* (PfENP) [65], in complex with cofactors, inhibitors, and/or fatty acyl substrate analogs. The crystal structure of InhA in complex with NAD+ and a C16 fatty acyl substrate supports the claim that, to produce fatty acyl substrates longer than C16 and thus precursors of mycolic acids (C16-C56), the substrate binding crevice of InhA must be able to accommodate fatty acyl substrates longer than C16 [61] . The MTB InhA substrate binding loop is longer and more flexible, creating a deeper binding crevice, than in either the *E. coli* or *Brassus napus* enoyl-ACP reductases, which prefer shorter chain substrates. This substrate binding loop is quite long in the PfENP, and by analogy with InhA, the PfENP would also be expected to bind very long chain fatty acids as substrates. Data from x-ray crystallography also confirm the complex action of isoniazid adducts: the cofactor NADH is modified by attachment of the isonicotinic-acyl group from isoniazid, which attaches through its carbonyl carbon to C4 of the nicotinamide ring, replacing the 4S hydrogen of NADH [60]. Residues implicated in binding NADH/NAD+, and residues of the active site as a whole, are highly conserved in all the enoyl-ACP reductase structures determined. Thus, compounds that do not require bioactivation would effectively circumvent the INH resistance mechanism. Diazoborines and triclosan represent InhA inhibitors that do not require bioactivation, but their utility has been limited due to poor solubility [66] and substantial toxicity [67].

To extend the range of pharmacophores from which to develop more potent and pharmacologically suitable inhibitors of enoyl-ACP reductases, Kuo *et al.* employed high-throughput screening methods against *M. tuberculosis* InhA [62]. The compound library was based on the indole-5 carboxylic acid structural unit, which was reacted with 86 different amines in limiting and equimolar ratios. The most potent analogs were amides of indole-5 carboxylic acid and 4-aryl-substituted piperazines. An array of piperazine analogs were then synthesized, from which two novel inhibitors of InhA, Genz-8575 and Genz-10850, were identified (Fig. **3**), and the crystal structure of the Genz-10850/InhA complex was determined. Further refinement of inhibitors is in progress, using the structure-activity relationships derived from these compounds.

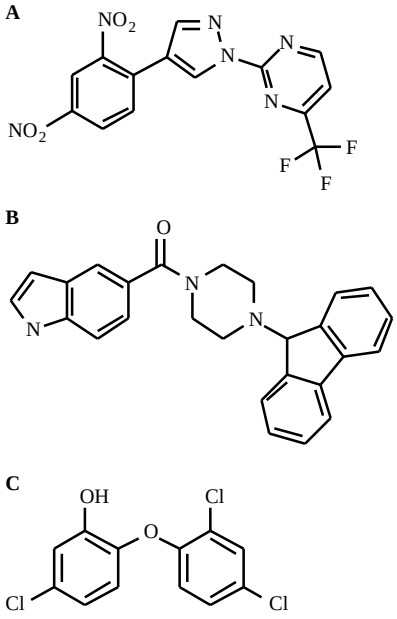

Fig. (3). *New enoyl-ACP reductase inhibitors.* Compounds discovered by Kuo *et al*. [62] from a high throughput combinatorial screen of recombinant *M. tuberculosis* InhA. A) Genz-8575; B) Genz-10850; C) triclosan

ETHIONAMIDE

After the discovery of isoniazid as a potent antitubercular agent, the subsequent search for structural analogs of isoniazid led to the discovery of ethionamide (ETA; 2-ethyl-4-pyridinecarbothioamide) [68, 69], an important second-line therapy for MDRTB. ETA has almost identical inhibitory effects on mycolic acid biosynthesis as INH, although *M. tuberculosis* strains resistant to one drug do not show cross resistance to the other, suggesting significant differences in their activation mechanisms [52]. It has been demonstrated that ETA is activated by S-oxidation before interacting with its cellular protein target, and the activated product, 4-pyridylmethanol, is remarkably similar in structure to the product formed by activation of INH by KatG. A wide variety of thiocarbonyl-containing compounds have been shown to undergo *in vitro* S-oxidation [70], further supporting this intermediate oxidation step in the metabolic activation of ETA.

deBarber *et al*. [71] have shown that overproduction of Rv3855 (EtaR), a putative regulatory protein from *M. tuberculosis*, confers ETA resistance, whereas overproduction of an adjacent, clustered monooxygenase (Rv3854c,

EtaA) confers ETA hypersensitivity. EtaA appears to be the activating enzyme responsible for thioamide oxidation and subsequent toxicity, because production of EtaA is negatively regulated by EtaR and correlates directly with [^{14}C] ETA metabolism. Rv3083 and Rv0565c are closely related homologs of EtaA (~50% sequence identity) encoded within the *M. tuberculosis* genome [28], and these belong to a family of related proteins, most of which are probable monooxygenases. The genome of *M. tuberculosis* also encodes for 20 cytochrome P450 containing oxygenases, which is the largest number identified within a single bacterial genome [72]. The abundance of oxidative enzymes in the *M. tuberculosis* genome may have arisen to promote survival by the bacterium in the face of attack by xenobiotic substances, and ETA susceptibility may also result from activation by an enzyme intended to aid detoxification.

There has been renewed interest in thiocarbamide drugs and new analogs [73], particularly in the developing world, because they are inexpensive [74]. Bioactivation of carbothioamides is believed to require S-oxidation for protein binding and enzyme inhibition [75, 76], and cross-resistance demonstrated among these second-line drugs [77, 78] suggests a common bioactivation mechanism for thiocarbonyl containing molecules. Although this shared chemistry complicates MDRTB therapy, it can be exploited as a general mechanism when developing novel antimycobacterials.

Targeting Amino Acid Biosynthesis

lysA

The MTB *lysA* gene (Rv1293) encodes the enzyme meso-diaminopimelate decarboxylase (DAPDC), a pyridoxyl-5'-phosphate (PLP)-dependent enzyme. In the final step of lysine biosynthesis, DADPC catalyzes the conversion of meso-diaminopimelic acid (DAP) to L-lysine. Because DADPC has been shown to be essential to *in vivo* viability of mycobacteria [79], it is an attractive drug target. The recently determined x-ray crystal structure of this enzyme reveals a structural homology to ornithine decarboxylase (ODC) [80, 81] (Fig. **4A**) from *Trypanosoma brucei* (TbODC) [82]. TbODC is a chemotherapeutic target for the treatment of African sleeping sickness, and the structure has provided the basis for the design of novel inhibitors, in particular those based on α-difluoromethyl ornithine (DFMO). DFMO is a suicide inhibitor of TbODC, which prevents the first committed step in polyamine biosynthesis required for cell growth and differentiation [83]. The active sites of LysA and TbODC are remarkably similar (Fig. **4B**). Using the structure of DFMO-bound TbODC as an model [82], we successfully simulated a docked complex of DFDAP and DAPDC from MTB [84], which strongly suggests that corresponding DAP analogs may be potential inhibitors for mycobacterial DAPDCs.

Targeting Protein Synthesis

def

The *def* gene in *M. tuberculosis* (Rv0429c) encodes for a peptide deformylase (PDF; EC 3.5.1.88). PDF catalyzes the deformylation reaction of the N-terminal fMET residue of newly synthesized proteins in eubacteria. The enzyme is essential for bacterial growth [85, 86] , making it an attractive target for the design of new antibiotics, although PDF orthologs have been found in organelles such as chloroplasts and mitochondria [87, 88]. A number of pharmaceutical companies are engaged in structure-based approaches to the design of bacterial PDF inhibitors [85, 86, 89].

PDF belongs to a class of metallohydrolases that utilize ferrous iron as the catalytic metal ion. The ferric form of the enzyme is inactive. Peptide deformylase sequences have significant similarity in three regions designated motif 1 (GΦGΦAAXQ), motif 2 (EGCΦS) and motif 3 (HEΦDH), where Φ is a hydrophobic amino acid. The C in motif 2, along with the two Hs in motif 3 coordinate the metal ion, with the fourth site occupied by a water molecule that hydrolyzes the amide bond. Catalytic efficiency has been shown to be strongly dependent on the identity of the bound metal. Substitution of the ferrous ion by nickel causes little loss in enzyme activity but increases stability, whereas substitution by zinc results in five orders of magnitude loss of activity [90]. The details of the catalytic metal center are known from crystal structures of several forms of PDF [90-92].

As a metalloprotease, PDF is an even more attractive drug target, because metalloproteases are among the best studied enzymes classes [93], and there has been a great deal of research using mechanism-based approaches to design inhibitors, which will not be discussed here. Availability of 3D structures of several PDF forms has made it possible to perform *de novo* structure-based inhibitor design in combination with mechanistic approaches, from which a generic inhibitor structure has been proposed (Fig. **5A**) [94]. Early transition state and substrate analog-based inhibitors showed no significant antibacterial activity, due either to lack of potency and/or inability to penetrate the bacterial cell [91, 95, 96]. Screening for inhibitors of *E. coli* PDF identified actinonin, a naturally occurring hydroxamic acid pseuodopeptide produced by *Actinomycetes* [97] as a potent inhibitor of Gram-positive bacteria, fastidious Gram-negative bacteria and plant PDFs [86, 98], but actinonin was relatively ineffective against eukaryotes, such as *Plasmodium falciparum* (Pf), as compared to *E. coli* [99]. A series of β-sulfonyl and β-sulfinylhydroxamic acid derivatives were then synthesized and evaluated *in vitro*, and crystallographic structures determined [89]. To understand the structural basis for the potent inhibition of PDF by actinonin, Chen *et al.* [86] built a model of actinonin bound to PDF using crystallographic coordinates for the uncomplexed Zn-PDF [100] and matrilysin, a matrix metalloprotease, bound to a hydroxamate-based inhibitor [101]. From the model and by analogy with previously developed inhibitors, it was concluded that the n-pentyl chain functions as the analog of methionine, the preferred P1' amino acid in PDF substrates [102]. The hydroxamate group acts as a chelating group, binding the catalytic metal ion, and this has been shown to be essential for antibacterial activity. A subsequent study of structure-activity relationships of actinonin analogs and other PDF inhibitors found that potent inhibition of several bacterial PDFs, including *E. coli*, *S. pneumoniae*, *C. pneumoniae, H. inflluenzae, M. pneumoniae, M. catarrhalis* and *B. subtilis*, could be achieved with low-molecular weight hydroxamate acid derivatives, possessing β-arylsulfonyl and

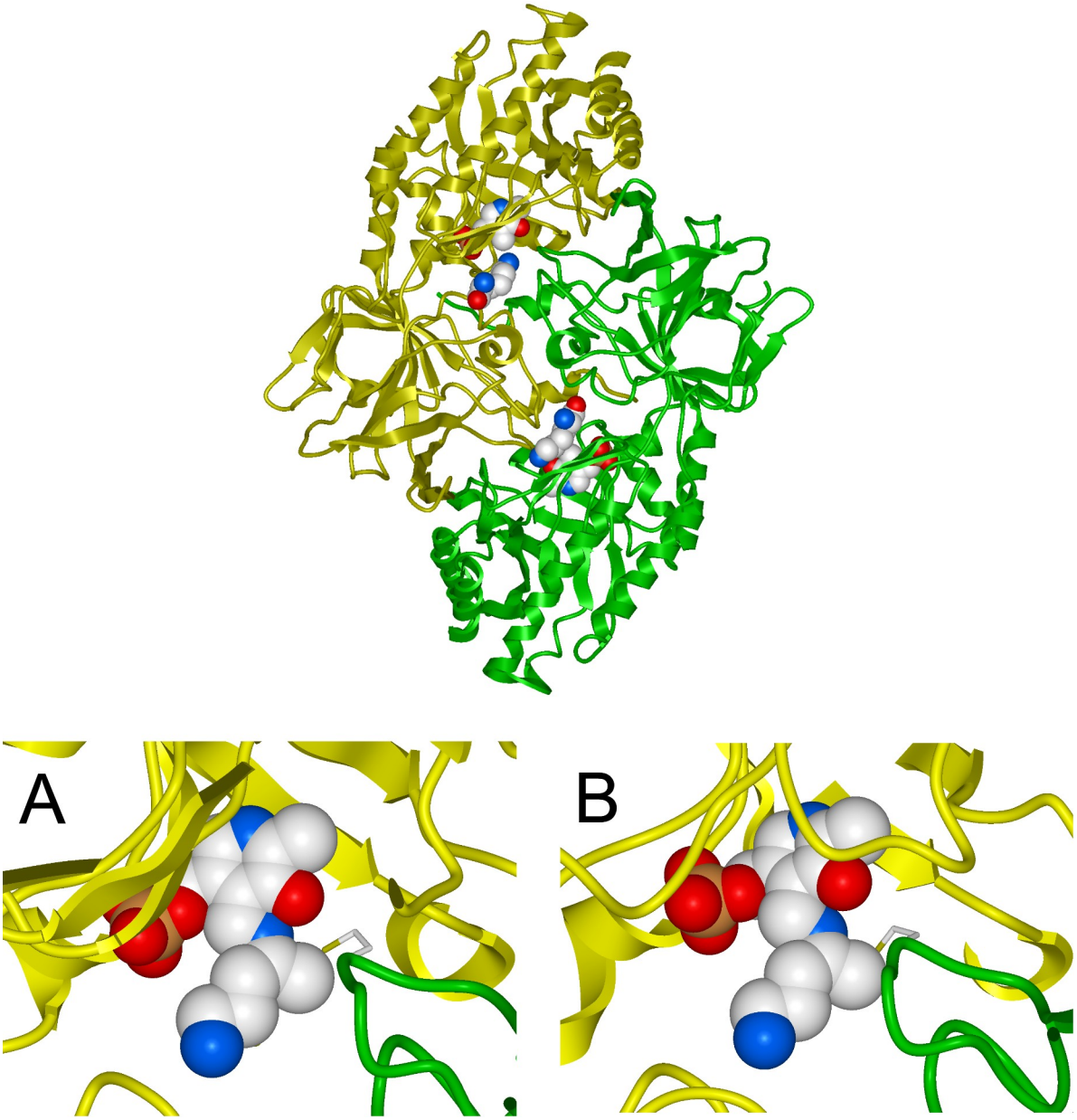

Fig. (4). *Structure of LysA from M. tuberculosis.* Top: Overall fold, as determined by Gokulan *et al*. [105], depicted as a ribbon diagram. Chains of the homodimers are colored yellow and green respectively. Lysine and PLP rendered in CPK. Bottom: Details of key residues interacting with substrates and inhibitors: A) MTB LysA and B) TbODC [77], bound to the suicide inhibitor DFMO. Protein colored by chain as previously. PLP and suicide inhibitor DFMO adduct rendered in CPK. Cysteine bound to adduct shown as sticks, colored by atom type. Docking performed with ICM-Pro [156]. Images rendered with ICM-Pro [156].

β-arylsulfinyl functionalities, although at relatively high concentrations [89]. The crystal structure of one lead compound in complex with *E. coli* Ni-PDF was determined, and the coordinates were used to model the predicted binding mode of a 24 compound library.

Clements and coworkers, using an extensive library of metalloenzyme inhibitors, identified a novel compound, BB-3497, as a potent and selective inhibitor of a number of clinically relevant bacterial pathogens [85]. BB-3497 has been shown to be active in murine systemic models of *S.*

aureus, and it is orally bioavailable. The x-ray crystal structures of both actinonin and BB-3497 complexed to Ni-PDF have now been determined, facilitating the design of novel inhibitors with improved pharmacokinetic and antibacterial properties [85]. In both structures, the active site nickel ion is pentacoordinated by the two oxygen atoms of the hydroxamate group in actinonin or the N-formyl-hydroxyoamine group of BB-3497, as well as the conserved cysteine and histidine side chains. The alkyl chains lie along the hydrophobic S1' pocket, and the P2' and P3' side chains are largely exposed to solvent, providing attractive sites for modification to improve molecular properties. The tight binding of BB-3497 and actinonin is achieved by mimicking the structural elements critical to the active site chemistry. Because these complexes do not represent the true structure of the transition state, a substantially weaker H-phosphonate transition state analogue inhibitor complex structure was determined, revealing a tetrahedrally coordinated metal center with a less complex hydrogen bonded network [91]. These differences have provided clues to the variation in activity between these inhibitor types.

Further validation of PDF as an *in vivo* target has been provided by Hackbarth and co-workers [94], who designed a new class of potent PDF inhibitor, which has N-alkyl urea at the P1' site. Compounds with MICs < 4μg/ml against Gram-positive and Gram-negative pathogens, have been identified, and IC_{50} for *E. coli* Ni-PDF was < 0.1mM. Structure-activity relationship analysis identified preferred substitutions to improve potency and reduce toxicity. The crystal structure of *E. coli* Ni-PDF with compound VRC4307 revealed that urea compounds bind in a position similar to that determined previously for succinate hydroxamates. Two compounds, VRC4232 and VRC4307, displayed moderate *in vivo* protective activity in a murine *S. aureas* septicemia model, although VRC4307 exhibited a poor pharmacokinetic profile. Matrix metalloproteases, whose catalytic metal sites are similar that of PDFs [100], were not inhibited by the urea compounds.

Based on the structure of the BB-3497 complex, Hu *et al*. [103] designed a cyclic bacterial PDF inhibitor, in which the nonyl group serves as the cross-linked P1' and P3' side chains, because molecular modeling suggested the nonyl group would be long enough to link the P1' Cα and the P3' amino group while preserving the extended backbone conformation. *In vitro* activity by the macrocycle was high against Co^{2+} substituted *E. coli* PDF, but when tested against the representative Gram-negative and Gram-positive bacteria, *E. coli* and *B. subtilis*, the MIC against *B. subtilis* was 2-4 μM, whereas against *E. coli*, the MIC was ~32 μM. The low activity against the latter is attributed to inefficient permeation of the bacterial outer membrane and/or removal from the cell *via* a bacterial efflux pump.

We have targeted MTB PDF for structure determination in our laboratory, but cloning, expression and purification have proved problematic, likely due to oxidative damage [104]. We are now attempting to exchange the catalytic iron for nickel or cobalt as active surrogates, which have been shown to be stable [90, 105]. In the absence of an experimental x-ray crystal structure, we have built a homology model of MTB PDF using the crystal structure of the *E. coli* enzyme [100], which shares 35% sequence identity with MTB PDF, as the template (Fig. **5B**). The MAS nonhydrolyzable peptide (Fig. **5C**), as well as several known inhibitors of *E. coli* PDF, were docked into the active site of MTB PDF, revealing similar modes of binding as seen in reported crystal structures. Differences in the structures of these drug targets, however, provide insights from which we propose new functionalities for the design of optimized inhibitors of the tuberculosis enzyme, which will be published elsewhere. A summary of promising published PDF inhibitors is provided in [106].

Kumar *et al*. have suggested that a putative mitochondrial *Homo sapiens* PDF (HsPDF) most likely folds and functions like *E. coli* and PfPDF [92]. However, because HsPDF shares only 23% identity with MTB PDF, differences between these drug targets will likely provide opportunities for the design of selective drug leads. The HsPDF cDNA has now been cloned, and a truncated form that lacks the N-terminal 58-amino-acid targeting sequence, and with Co^{2+} at the metal center, was overexpressed in *E. coli* [107]. The recombinant enzyme is catalytically active in deformylating N-formylated peptides, shares many of the properties of bacterial PDF, and is strongly inhibited by known PDF inhibitors. HsPDF is much less active than bacterial orthologs, however, due in part to mutation of a highly conserved leucine residue, explaining perhaps the apparent lack of deformylation in mammalian mitochondria. PDF inhibitors had no detectable effect on two different human cell lines. It is likely then, that HsPDF is an evolutionary remnant with no functional role in protein formylation/deformylation, validating the potential of PDF as an antibacterial drug target. However, evidence of actinonin-resistant mutants [108, 109], as well as reports that formylation is not essential for initiation of protein synthesis in all eubacteria [110], have raised additional questions about the validity of PDF as a target for broad spectrum antibacterial chemotherapy. Most mutants have been shown to be defective in methionine formylation, which bypasses PDF activity, and they are no longer viable [111]. It is not known, however, whether formylation is an absolute requirement for the initiation of protein synthesis in *M. tuberculosis*.

New Drug Targets in Cell Wall Biosynthesis

rmlC

L-rhamnose, a 6-deoxyhexose, is a naturally occurring carbohydrate found in the cell wall of many pathogenic bacteria [112, 113]. In many Gram-negative bacteria, L-rhamnose is one of the important residues of the O-antigen lipopolysaccharide, a key determinant for virulence, whereas in Gram-positive bacteria such as streptococci, L-rhamnose is a component of the cell wall or capsule. In mycobacteria, L-rhamnose is a component of the arabinogalactan, which attaches the lipid mycolic acid layer to the peptidoglycan layer [113]. Because these bacterial cell wall components are not found in eukaryotic cell membranes, inhibitors of the formation of arbinogalactan, such as ethambutol, can stop cell growth and have been shown to be effective antitubercular drugs [114].

L-rhamnose is derived from a glucose scaffold in four steps, starting with glucose-1-phosphate and deoxythymidine

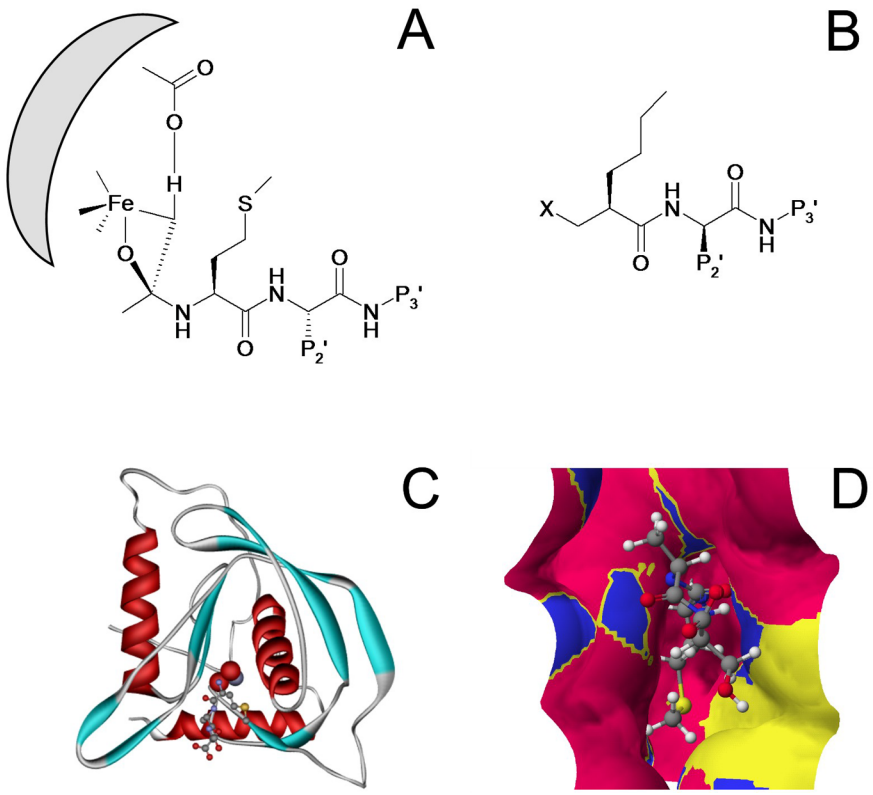

Fig. (5). *M. tuberculosis peptide deformylase.* A) Transition state of PDFs characterized by a formylmethionyl peptide bound to a metal ion (Fe^{2+}, Ni^{2+} or Co^{2+} are catalytic centers). B) Generic inhibitor containing a functional group, X, that typically chelates the metal. A generic inhibitor contains a X, that typically chelates the metal ion, while the remainder of the molecule mimics the substrate methionyl peptide. C) Ribbon diagram of the MTB PDF homology model based upon the *E. coli* PDF template [100], bound to the tripeptide product MAS (ball and stick). Metal ion and bound water shown in CPK. Rendered with WeLabViewerLite 4.5© D) Adjacent surface of the MTB PDF active site highlighting nature of residues forming binding pocket. Red denotes hydrogen acceptor groups, blue hydrogen donor groups, and yellow hydrophobic groups. Docking and adjacent surface computed with BioMedCaChe v6.0a1©.

triphosphate, to yield deoxythymidine diphospho-L-rhamnose. The enzymes in the rhamnose pathway (Fig. **6**) are glucose-1-phosphate thymidylyltransferase (RmlA, EC 2.7.7.24), dTDP-D-glucose 4, 6-dehydratase (RmlB, EC 4.2.1.46), dTDP-6-deoxy-D-xylo-4-hexulose reductase (RmlC, EC 5.1.3.13) and dTDP-6-deoxy-L-xylo-4-hexulose reductase (RmlD, EC 1.1.1.113). RmlA couples the glucose-1-phosphate moiety to deoxythymidine triphosphate, RmlB oxidizes the 4' hydroxyl and dehydrates the 6' hydroxyl, RmlC inverts the 3' and 5' hydroxyls, creating an unstable ring structure which flips, and RmlD reduces the 4' ketone to form the end product, dTDP rhamnose. These proteins are highly conserved among bacteria [115, 116], and as might be expected, conclusions drawn from the structure of an enzyme from one bacterium will have implications for design of inhibitors of the protein from another.

The crystal structures of all four enzymes in the rhamnose pathway have been determined with and without substrates and cofactors bound: RmlA from *Pseudomonas aeruginosa* [117], *Salmonella enterica (PDB ID: 1MP3),* and *Methanobacterium thermoautotrophicum (PDB ID: 1LVW)*; RmlB from *Salmonella typhimurium* [118] and *Streptococcus suis* [119]; RmlC from *Methanobacterium thermoautotrophicum* [120], *Salmonella typhimurium* [121], *Streptococcus suis* [122] and *Mycobacterium tuberculosis* [157]; and RmlD from *Salmonella typhimurium* [123]. The structures of RmlB and RmlD belong to the classical NAD(P)-binding Rossman fold superfamily, RmlA belongs to the nucleotide-diphosphotransferase super-family, and RmlC has a novel structure, the RmlC-like cupin structure, a double-stranded beta helix. The *rmlC* gene (Rv3465) is believed to be the best drug target in the pathway, because the encoded protein is highly specific, it is structurally unique, and it does not require a cofactor.

In an attempt to find new inhibitors of the enzymes in the essential rhamnose pathway, a virtual library of 2, 3, 5 tri-substituted-4-thiazolidinones was created, docked sequentially into the active site cavity of the *M. tuberculosis* RmlC and consensus scored [124] . The top 5% were slated for synthesis, and 94 compounds were successfully synthesized and tested. Of these, 30 (32%) have ≥50% inhibitory activity (at 20μM) in a coupled rhamnose synthetic assay, and seven

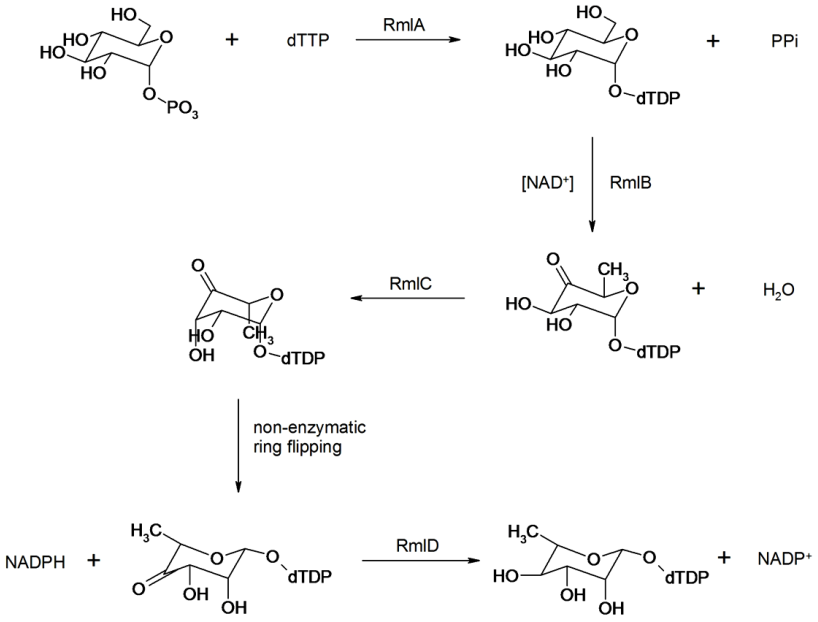

Fig. (6). The dTDP rhamnose biosynthetic pathway in *M. tuberculosis*. *rmlC* encodes for dTDP-6-deoxy-D-4-hexulose, the third enzyme in the dTDP rhamnose pathway. L-rhamnose is derived from a glucose scaffold in four steps, beginning with glucose-1-phosphate (upper left) and deoxythymidine triphosphate (dTTP). RmlC inverts the 3' and 5' hydroxyls, producing an unstable ring structure, which flips prior to the final catalytic step.

have modest MIC values (≤ 50μg/mL) against whole-cell *M. tuberculosis* H37Rv. Although the strongest lead inhibitory compound in the enzymatic assay (Fig. **7**), had little effect on bacterial growth due to poor penetration or bacterial metabolism, the results of this study again support the proposed hypothesis that the thiazolidinone scaffold is capable of acting as a diphosphate mimic, and they suggest that it is possible to design specificity for this class of inhibitor against enzymes in the same pathway. More recently, other potential RmlC inhibitors have been discovered [125], and further studies to determine crystal structures of RmlC/inhibitor complexes and create new combinatorial libraries are underway in several laboratories. Virtual ligand screening against MTB RmlC in our laboratory has provided additional insights into the structure-activity relationships of these potential RmlC inhibitors [157]. These case studies exemplify the successful use of a structural bioinformatics approach to develop and prioritize a combinatorial library.

glf

UDP-galactofuranose (UDP-Galf) residues are vital components of the arabinogalactan that connects the peptidoglycan and mycolic acids in the mycobacterial cell wall. The critical location of galactofuran between the peptidoglycan and mycolic acids points to an essential role in bacterial growth [126]. Further evidence for this role is supported by the action of ethambutol, an effective antitubercular drug, which inhibits the formation of arabinan [127]. The *embAB* genes of mycobacteria encode for arabinosyltransferases involved in cell wall arabinan biosynthesis, of which embA is the target of the antimycobacterial drug, ethambutol [128]. Unfortunately, the 3D structures of these enzymes are not known, as they are membrane proteins, and the mechanism of action of ethambutol has yet to be elucidated.

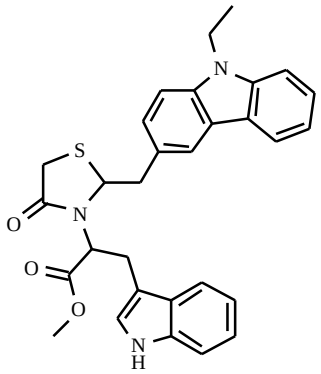

Fig. (7). *Substituted thiazolidinone showing the best inhibitory activity against RmlC in M. tuberculosis*. At 20 mM, this compound showed 48% RmlC inhibition and MIC *vs. M. tuberculosis* H37Rv > 200 μg/L [124].

The enzyme responsible for converting UDP-galactopyranose (UDP-Galp) to UDP-Galf is UDP-galactopyranose mutase, encoded by the *glf* gene (Rv3809c). The crystal structure of UDP-Galf from *E. coli* has been solved, revealing a novel structure [129] and putative mechanism [130]. The mutase is a mixed α/β protein, dimeric in the crystal, consistent with solution scattering experiments (Fig. **8**). Mutase incorporates flavin adenine dinucleotide (FAD) in domain 1, which is similar to other FAD-binding domains in proteins. Two other domains (2, 3) are structurally unique. A large cleft between domains 1 and 2 forms the FAD binding site. Based upon the crystal structure, Sanders *et al*. proposed that the right side of the cleft, being positively charged and containing many of the conserved residues in mutase, would be the site of UDP-galactose binding [130]. Docking of UDP-galactose to the structure suggests that uridine ring

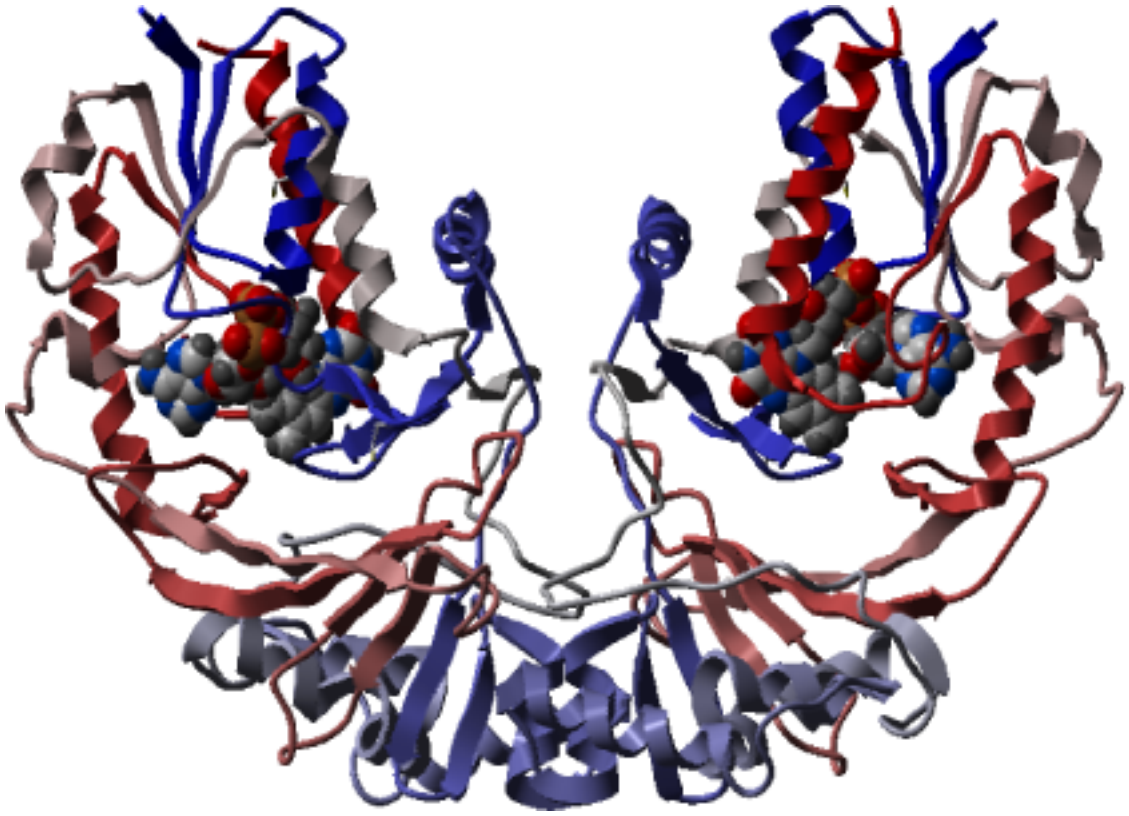

Fig. (8). *Structure of E. coli UDP-Galf.* Ribbon diagram of the homodimer, colored N-terminal (blue) to C-terminal (red). Viewing the monomer to the right, domain 1, at top of the figure, includes the αβα Rossman fold motif. Domain 2 consists primarily of a five helix bundle at the bottom of the figure, and which is connected to domain 1 by domain 3, the six-stranded antiparallel β-sheet in the middle of the figure. FAD, bound in the large cleft formed by domains 1 and 2, is rendered as CPK. Coordinates determined by Sanders *et al*. [130]. Image rendered with ICM-Pro [156].

should stack against Trp 156, a feature noted in other UDP-Gal binding proteins.

Fullerton and co-workers performed a potentiometric analysis [131], which showed that the neutral flavosemiquinone ($FADH\cdot$) is stabilized by bound substrate, and that the fully reduced flavin is the anionic $FADH^-$ rather than $FADH_2$ at pH 7.0. In the UDP-Gal docked model, the carbohydrate ring is adjacent to the isoalloxazine ring of the FAD, and conserved residues R247 and R278 bind the phosphate bridges of the nucleotide. Potentiometric analysis supports this mode of binding. A proposed catalytic mechanism would require that the UDP be transiently separated from galactose by breakage of the glycosidic bond to account for isotopic exchange observed during catalysis [132]. Two mechanisms leading to the observed products have been put forward. One involves a single-electron transfer from the anionic quinone to the oxocarbonium, producing an anomeric radical and forming the neutral semiquinone [131]. This avoids stable intermediates, proceeds rapidly and reversibly, and has been observed previously in the chemistry of furanosyl and pyranosyl functionalities [133] . Kiessling and coworkers now propose that a reduced anionic form of the flavin forms an imine adduct with the galactopyranose substrate by nucleophilic addition, leading to conversion of the substrate to its galactofuranose form [134]. Several small organic compounds have also been identified that inhibit UDP-Galf from tuberculosis and pneumonia bacteria at micromolar levels, providing leads to new antimycobacterial agents, as well as facilitating studies of galactofuranose recognition and incorporation [134].

Scherman *et al*. [135] have recently developed a microtiter plate assay for MTB UDP-Galf, based on the release of titrated formaldehyde by periodate from UDP-galactofuranose but not UDP-galactopyranose. Using this assay, a putative uridine-based enzyme inhibitor, 320KAW73, has been identified from a chemical library. The structure of the MTB UDP-Galf has recently been determined, but the atomic coordinates have not yet been published [135]. We have docked the inhibitor 320KAW73 to an excellent quality homology model of MTB UDP-Galf, based upon the *E. coli* enzyme, and find that the preferred mode of binding is consistent with the model proposed by Sanders *et al*. [130] for the binding of UDP-Gal. In the case of 320KAW73 binding, the essential phenylsulfonyl diphosphate analog spacer interacts with conserved arginines and the carbohydrate moiety is adjacent to the isoalloxazine ring. Unfortunately, this inhibitor was not active against whole *M. tuberculosis*, most likely due to poor absorption [135]. Nevertheless, this drug lead, as in the RmlC case study, provides valuable structural information for the design of

compounds possessing functionalities that potentially exhibit better deliverability, and the results of modeling underscore the importance of understanding the structural basis of the protein-ligand interaction. Further screening with a commercial library of drug-like compounds from Nanosyn, Menlo Park, CA, is in progress [135].

Targeting Genes Implicated in Persistence

icl

The enzymes isocitrate lyase (*icl*; Rv0467) and malate synthase (*glcB*; Rv11837c) together form the glyoxalate shunt, bypassing the CO_2 - generating steps of the tricarboxylic acid (TCA) cycle, which provides a way for acetate (from fatty acids) to replenish the TCA cycle. ICL catalyzes the first committed step in the pathway, facilitating net carbon gain by diverting acetyl-CoA from β-oxidation of fatty acids into the glyoxalate shunt. The shunt allows *M. tuberculosis* and other bacteria to "lunch on lipids", synthesizing carbohydrates from fatty acids. McKinney *et al*. have shown that ICL is activated in the latent state and is important for long-term survival [136].

Given its potential as a drug target against persistent infections, Sharma *et al*. solved the structure of ICL without ligand and in complex with two known ICL inhibitors, 3-nitropropionate and 3-bromopyruvate [137]. Structural knowledge about the active sites of ICL from *M. tuberculosis* and *E. coli* [138], as well as related members of this protein family, has provided insights into the catalytic mechanism of ICL and structural basis for substrate specificity, and the search for additional inhibitors based upon these molecular scaffolds is in progress.

pyrR

The *de novo* synthesis of pyrimidines is universal, the pathway consisting of six enzymatic steps leading to the formation of UMP, which is then converted to UTP, CTP, dCTP and dTTP. PyrR is a protein that regulates expression of genes and operons of pyrimidine nucleotide biosynthesis (*pyr* genes) in many bacteria. In *M. tuberculosis*, the pyrimidine biosynthesis genes (*pyr*) are located on a single operon [28]. PyrR, encoded by the *pyrR* gene (Rv1379) acts by binding to specific sequences on *pyr* mRNA, causing transcriptional attenuation when intracellular levels of uridine nucleotides are elevated. The binding of PyrR to mRNA is dependent on the formation of a PyrR-UMP complex. The protein may also be a uracil phosphoribosyltransferase (UPRTase) [139]. *pyrR* has been shown to be upregulated during hypoxic stress, characteristic of the environment found in the granuloma harboring *M. tuberculosis* [140, 141]. Because pyrimidine biosynthesis is an essential step in the progression of tuberculosis [142], *pyrR* is an attractive antitubercular drug target.

The structure of MTB PyrR (PDB ID: 1W30) has been solved in our laboratory (unpublished) by molecular replacement using a homology model based on the structure of the enzyme from *Bacillus subtilis* as the probe [143], which shares 49% sequence identity. Simulated docking of UMP reveals a predicted binding mode that is similar to UPRTases (Fig. **9**). The UMP binding site of the MTB PyrR

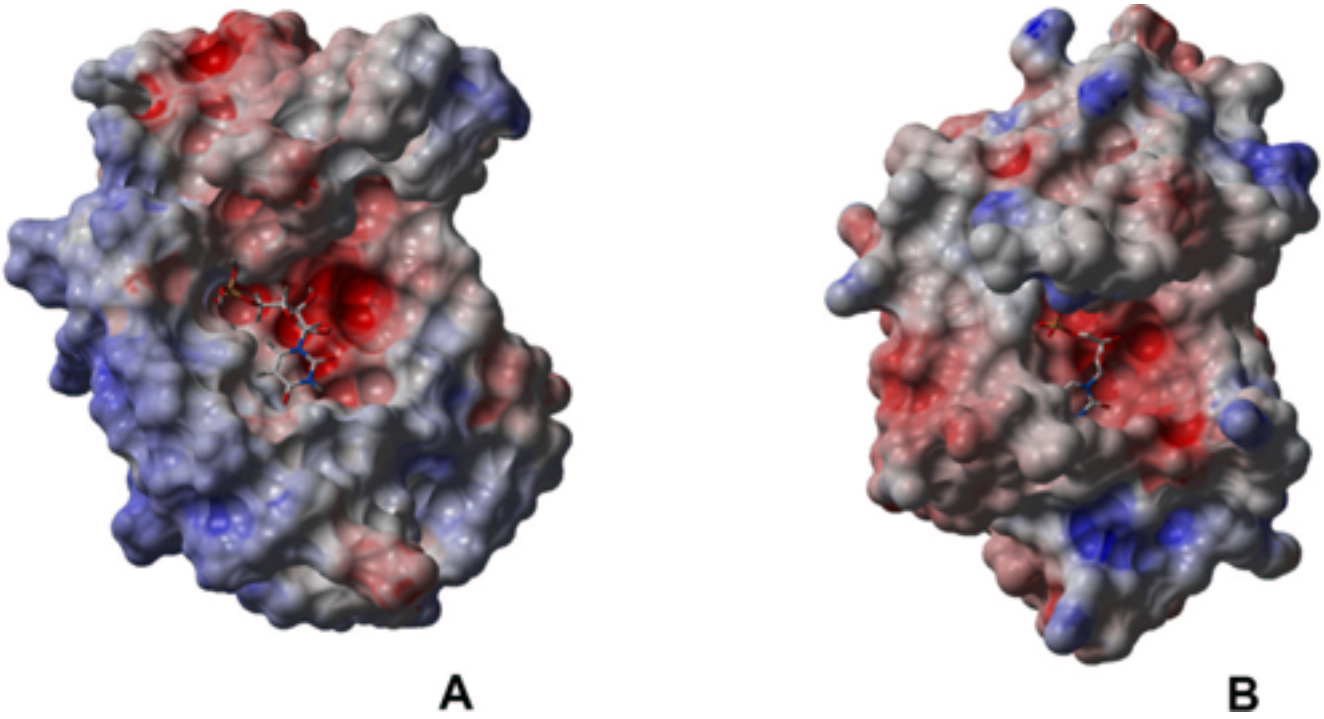

Fig. (9). *UMP binding sites in M. tuberculosis pyrR and B. caldolyticus UPRTase*. UMP binds in a similar manner to MTB pyrR (A) as seen in the crystal structure of *B. caldolyticus* UPRTase (B). Interesting differences in the size, shape and charge disposition of the binding pocket may be exploited to design selective inhibitors of PyrR. Docking of UMP to MTB PyrR performed, and images rendered with, ICM-Pro [156]. Structure of MTB PyrR to be published.

contains many of the conserved residues in PyrRs and UPRTases, including a conserved aspartate involved in binding the ribosyl moiety of UMP. However, significant differences in the size, shape and charge disposition of the binding pocket from the active site in the UPRTases from *Bacillus caldolyticus* and *Toxoplasma gondii*, whose 3D structures are known [144, 145], help to explain the weak catalytic activity of the TB enzyme, and these features may be exploited to design selective ligands of MTB PyrR. Virtual screening of various pyrimidine nucleoside analogs against MTB PyrR, has revealed a number of potential drug leads. Efforts are underway in our laboratory to determine crystal structures of MTB PyrR in complex with several of these compounds.

Inhibiting the Folate Pathway

dfrA

Although the ideal antimicrobial drug will target a protein or proteins unique to the microbial system, some of the more effective drugs actually act on a universal cellular component. Structure-guided approaches then benefit from analysis and comparison with mammalian homologs, which help to develop specificity and predict toxicity. DHFR (DHFR; EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7, 8-dihydrofolate to 5, 6, 7, 8-tetrahydrofolate, which is involved in a variety of biochemical functions involving single-carbon transfers [146, 147]. The reduced form of folate is a precursor of cofactors necessary for the synthesis of thymidylate and purine nucleotides required for DNA and RNA biosynthesis, as well as methionine, serine and glycine required for peptide biosynthesis.

Because inhibition of DHFR results in cell death, this enzyme has been the subject of intense study as a drug target for chemotherapy in neoplastic and autoimmune diseases, as well as for fungal, protozoal and bacterial infections [148]. Investigations have been accompanied by structure-guided approaches to analyze differences in the active site regions of human and bacterial DHFR to understand better the structural features of these enzymes that control ligand binding and selectivity. Studies have revealed that novel compounds from diverse chemical classes are capable of inhibiting DHFRs. Molecular modeling in combination with sequence profiles and crystal structure data has produced a series of antifolate compounds whose binding and selectivity can be rationalized [149, 150].

The three-dimensional structures of the *M. tuberculosis* DHFR (*dfrA*; Rv2763) unbound, in binary complex with NADP, and in ternary complex with NADP and one of three inhibitors, have revealed structural features that may be exploited for the design of novel tuberculosis drugs [151]. The three inhibitors are the anticancer drug methotrexate, the antimicrobial trimethoprim, and the antimalarial Br-WR99210. Despite sequence identity as little as 20% among DHFRs from different species (26% between MTB and human), the overall structure of the enzymes is the same: a central β-sheet, with a left-handed twist of about 130°, flanked by four α-helices [152]. However, these high resolution MTB structures (1.7Å – 2.0Å) reveal interesting differences between host and pathogen enzymes in the vicinity of the NADP. In each of the three inhibitor complexes, near the aminopterin ring of methotrexate, the aminopyrimidine ring of trimethoprim, and the aminotriazine ring of Br-WR99210, a glycerol molecule is bound in a region that, by contrast, is filled with hydrophobic residues in human DHFR (Fig. **10**). In addition, the binding site near the N6 of NADP is more hydrophobic in the MTB DHFR than the human. These differences may be exploited to design more effective inhibitors of MTB DHFR, perhaps through addition of neutral and hydrophobic functionalities to the existing drug frameworks. Such inhibitors would be more selective for MTB DHFR (and less toxic to the host), have higher affinity for the MTB enzyme, and would also perhaps be more permeable to the mycobacterial cell envelope, thus having enhanced deliverability.

CONCLUDING REMARKS

A structural bioinformatic approach to drug discovery exploits computational techniques to examine the flow of information from genome to structure to function. In post-genomics, structure is often the first and easiest to obtain information needed to rationalize function, and possibilities for successful structure-based ligand design are enormous. We can use bioinformatics to make rational hypotheses and plan experiments based on structural modeling and analysis. The outcome of this approach will be a set of critically evaluated models of putative MTB drug targets and a corresponding series of potential lead compounds, to be followed by target validation experiments, such as IC_{50} and MIC measurements.

Of particular interest will be finding drugs that target clinically latent bacteria, the best example being *M. tuberculosis*. When antimicrobials are added to cultures of latent bacteria, there are numerous changes in gene expression at the mRNA level, yet only a few of these are known to be related to antibiotic resistance mechanisms [153]. Thus, clinically latent bacteria may be highly adaptable. In patients with active tuberculosis, bacteria are multiplying and quickly killed by antimicrobial agents, although chemotherapy is required for six months, because latent bacteria are tolerant to existing antimicrobials [154]. Moreover, the genetics of antimicrobial tolerance are still poorly understood, and the molecular basis of this tolerance should be examined using gene knockouts, genomics and proteomics. The result may be the development of new antimycobacterial drugs, as well as prolonging the life of existing drugs, which may be used in combination with new chemotherapeutics. Chemotherapy would be more efficacious, shortening the course of chemotherapy and decreasing drug costs, patients would comply with drug regiments, and the potential for developing drug resistance would be lessened.

However, as we have seen in several of the case studies described, success in discovering potential new therapeutics using the informatic, structure-guided approach does not necessarily mean that we have found an effective therapeutic against whole *M. tuberculosis.* We may also have to consider the permeability barrier of the mycobacterial envelope and new routes for drug delivery, such as exploiting or modulating bacterial transport systems [155]. Computer modeling may then be used to design a "Trojan horse" drug, by which a lead compound is conjugated to a normally transported

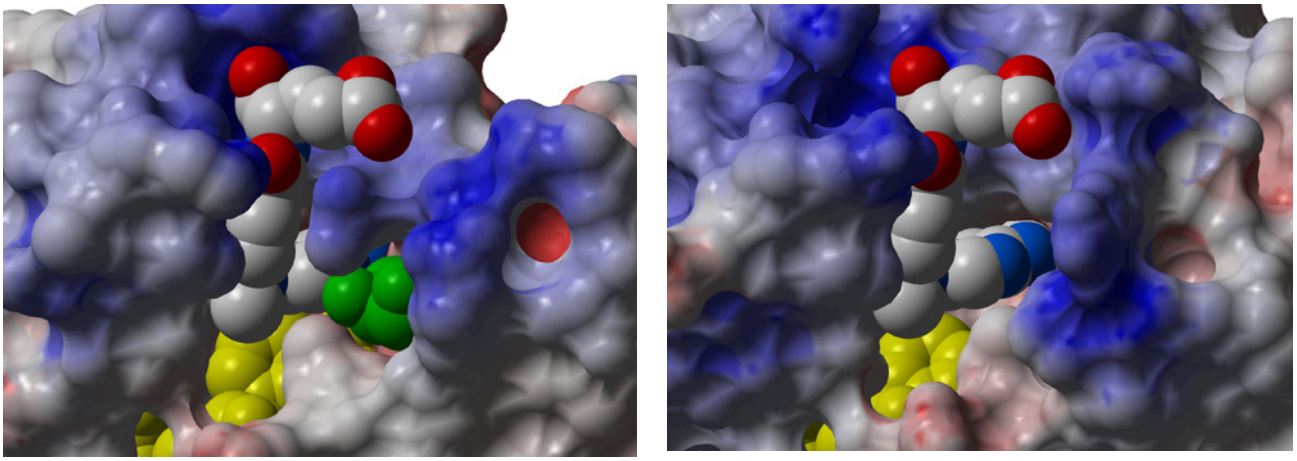

Fig. (10). *Methotrexate-bound DHFR from M. tuberculosis and Homo sapiens*. A) Active site of MTB DHFR shown with electrostatic potential surface, and bound methotrexate and glycerol (green) rendered in CPK; B) Active site of human DHFR shown with electrostatic potential surface, and bound methotrexate docked *in silico*. NADP shown in yellow CPK. The active site of MTB DHFR is larger and less hydrophobic, providing prospects for the design of novel chemotherapeutics with increased selectivity and affinity, and reduced toxicity.

substrate, or perhaps a biomimetic transport system for directed delivery. Alternatively, as novel compounds with enhanced antimycobacterial activity are developed using informatic strategies, it will be possible to produce a library of functionalities known to exhibit 'good' deliverability, and this library can be utilized in future to optimize lead structures.

REFERENCES

References 158-160 are related articles recently published in Current Pharmaceutical Design.

[1] Baker D, Sali A. Protein structure prediction and structural genomics. Science 2001; 294: 93-6.

[2] Lipinski CA. In BIOTEC, PDD symposium, AAPS: Miami, FL, 1995.

[3] Lipinski CA. Drug-like properties and the causes of poor solubility and permeability. J Pharmacol Toxicol 2000; 44: 235-49.

[4] Walters WP, Ajay Murcko MA. Recognizing molecules with drug-like properties. Curr Opin Chem Biol 1999; 3: 384-87.

[5] Oprea TI. Property distribution of drug-related chemical databases. J Comput Aided Mol Des 2000; 14: 251-64.

[6] Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 2001; 46: 3-26.

[7] Blundell TL, Mizuguchi K. Structural genomics: an overview. Prog Biophys Mol Biol 2000; 73: 289-95.

[8] Yon J, Jothi H. High throughput structural genomics and proteomics: where are we now? Targets 2003; 2: 201-7.

[9] WHO. Tuberculosis on the increase. World Health Forum 1993; 14: 1-94.

[10] Smith I. *Mycobacterium tuberculosis* and molecular determinants of virulence. Clin Microbiol Rev 2003; 16: 463-96.

[11] Davies J. Bacteria on the rampage. Nature 1996; 383: 219-20.

[12] Laing RO, McGoldrick K. In World Tuberculosis Day at Boston University School of Public Health: Boston, MA, 2000.

[13] Dye C, Williams BG, Espinal MA, Raviglione MC. Erasing the world's slow strain: Stategies to beat multidrug-resistant tuberculosis. Science 2002; 295: 2042-46.

[14] Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, *et al*. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998; 393: 537-44.

[15] Tekaia F, Gordon S, Garnier T, Brosch R, Barrell B, Cole S. Analysis of the proteome of *Mycobacterium tuberculosis* in silico. Tuber Lung Dis 1999; 79: 329-42.

[16] Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, *et al*. inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis.* Science 1994; 263: 227-30.

[17] Zhang Y, Heym B, Allen B, Young D, Cole ST. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 1992; 358: 591-93.

[18] Sensi P. History of the development of rifampin. Rev Infect Dis 1983; 3: 402-6.

[19] Dougherty TL, Barrett JF, Pucci MJ. Microbial genomics and novel antibiotic discovery: new technology to search for new drugs. Curr Pharm Des 2002; 8: 1119-35.

[20] Diekema DI, Jones RN. Oxazolidinones: a review. Drugs. 2000; 59: 7-16.

[21] Schapira M, Raaka BM, Das S, Totrov M, Zhou Z, Wilson SR, *et al*. Discovery of diverse thyroid hormone receptor antagonists by high-throughput docking. Proc Natl Acad Sci USA 2003; 100: 7354-59.

[22] Abagyan R, Totrov M, Kuznetsov D. ICM: a new method for protein modeling and design. Applications to docking and structure prediction from the distorted native conformation. J Comp Chem 1994; 15: 488-506.

[23] Schapira M, Abagyan R, Totrov M. Nuclear hormone receptor targeted virtual screening. J Med Chem 2003; 46: 3045-59.

[24] Abagyan R, Totrov M. High-throughput docking for lead generation. Curr Opin Chem Biol 2001; 5: 375-82.

[25] Muegge I, Rarey M. Small molecule docking and scoring. Rev Comp Chem 2001; 17: 1-60.

[26] Halperin I, Ma B, Wolfson H, Nussinov R. Principles of docking: An overview of search algorithms and a guide to scoring functions. Proteins 2002; 47: 409-43.

[27] Kissinger CR, Gehlhaar DK, Smith BA, Bouzida D. Molecular replacement by evolutionary search. Acta Crystallogr 2001; 57: 1474-9.

[28] Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, *et al*. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998; 393: 537-44.

[29] Camus JC, Pryor MJ, Medigue C, Cole ST. Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. Microbiology 2002; 148: 2967-73.

[30] Cole ST, Eigelmeier K, Parkill J, James KD, Thomson NR, Wheeler PR, *et al*. Massive gene decay in the leprosy bacillus. Nature 2001; 409: 1007-11.

[31] Garnier T, Eigelmeier K, Camus JC, Medina N, Mansoor H, Pryor MJ, *et al*. The complete genome sequence of *Mycobacterium bovis*. Proc Natl Acad Sci USA 2003; 100: 7877-82.

[32] Brennan MJ, Delogu G. The PE multigene family: a 'molecular mantra' for mycobacteria. Trends Microbiol 2002; 10: 246-8.

[33] Gamieldien J, Ptitsyn A, Hide W. Eukaryotic genes in *Mycobacterium tuberculosis* could have a role in pathogenesis and immunomodulation. Trends Genet 2002; 18: 5-8.

[34] Westbrook J, Feng Z, Chen L, Yang H, Berman HM. The Protein Data Bank and structural genomics. Nucleic Acids Res 2000; 31: 489-91.

[35] Sanchez R, Sali A. Comparative protein structure modeling. Introduction and practical examples with modeller. Methods Mol Biol 2000; 143: 97-129.

[36] Brooijmans N, Kuntz ID. Molecular recognition and docking algorithms. Annu Rev Biophys Biomol Struct 2003; 32: 335-73.

[37] Chen YZ, Zhi DG. Ligand-protein inverse docking and its use in the computer search of protein targets of a small molecule. Proteins 2001; 43: 217-26.

[38] Fernandez-Recio J, Totrov M, Abagyan R. ICM-DISCO docking by global energy optimizaton with fully flexible side-chains. Proteins 2003; 52: 113-7.

[39] Gray JJ, Moughon S, Wang C, Schueler-Furman O, Kuhlman B, Rohl CA, *et al*. Protein-protein docking with simultaneous optimization of rigid-body displacement and side-chain conformations. J Mol Biol 2003; 331: 281-99.

[40] Chen YZ, Zhi DG. Ligand-protein inverse docking and its potential use in the computer search of protein targets of a small molecule. Proteins 2001; 43: 217-26.

[41] Mendez R, Leplae R, De Maria L, Wodak SJ. Assessment of blind predictions of protein-protein interactions: current status of docking methods. Proteins 2003; 52: 51-67.

[42] Kremer L, Baulard AR, Besra GS. In: Hatfull G, Jacobs WR Jr Eds, Molecular Genetics of Mycobacteria. Washington DC, ASM Press, 2000; 173-90.

[43] Daffe M, Draper P. The envelop layers of mycobacteria with reference to their pathogenicity. Adv Microb Physiol 1998; 39: 131-203.

[44] Crick DC, Brennan PJ. Antituberculosis drug research. Curr Opin Anti-Inf Invest Drugs 2000; 2: 154-63.

[45] Schroeder EK, de Souza N, Santos DS, Blanchard JS, Basso LA. Drugs that inhibit mycolic acid biosynthesis in *Mycobacterium tuberculosis*. Curr Pharm Biotech 2002; 3: 197-225.

[46] Brennen PJ, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem 1995; 64: 29-63.

[47] Belanger AE, Inamine JM. In: Hatfull G, Jacobs WR Jr Eds, Molecular Genetics of Mycobacteria. Washington DC, ASM Press, 2000; 191-202.

[48] Bernstein J, Jambor WP, Lott WA, Pansy F, Steinberg BA, Yale HL. Chemotherapy of experimental tuberculosis. VI. Derivatives of isoniazid. Am Rev Tuberc 1953; 67: 354-65.

[49] Bernstein J, Jambor WP, Lott WA, Pansy F, Steinberg BA, Yale HL. Chemotherapy of experimental tuberculosis. VII. Heterocyclic acid hydrazides and derivatives. Am Rev Tuberc 1953; 67: 366-75.

[50] Middlebrook G. Isoniazid-resistance and catalase activity of tubercle bacilli; a preliminary report. Am Rev Tuberc 1954; 69: 471-72.

[51] Johnsson K, Schultz PG. Mechanistic studies of the oxidation of isoniazid by the catalase peroxidase from *Mycobacterium tuberculosis*. J Am Chem Soc 1994; 116: 7425-26.

[52] Johnsson K, King DS, Schultz PG. Studies of the mechanism of action of isoniazid and thionamide in the chemotherapy of tuberculosis. J Am Chem Soc 1995; 117: 5009-10.

[53] Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, van Embden JD. Characterization of the catalase-peroxidase gene (katG) and inhA locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restructed array of mutations associated with drug resistance. J Infect Dis 1996; 173: 196-202.

[54] Bloch K. Control mechanisms for fatty acid synthesis in *Mycobacterium smegmatis*. Adv Enzymol Relat Areas Mol Biol 1977; 45: 1-84.

[55] Waller RF, Keeling PJ, Donald RG, Striepen B, Handman E, Lang-Unnasch N, *et al*. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. Proc Natl Acad Sci USA 1998; 95: 12352-57.

[56] Geiter LJ. Targeted tuberculin testing and treatment of latent tuberculosis infection. Am J Respir Crit Care Med 2002; 4: 221-7.

[57] Wayne LG, Lin KY. Glyoxalate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. Infect Immun 1982; 37: 1042-49.

[58] Wayne LG, Hayes LG. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect Immun 1996; 64: 2062-69.

[59] Dessen A, Quemard A, Blanchard JS, Jacobs WR Jr, Sacchettini JC. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science 1995; 267: 1638-41.

[60] Rozwarski DA, Grant GA, Barton DH, Jacobs WR Jr, Sacchettini JC. Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. Science 1998; 279: 98-102.

[61] Rozwarski DA, Vilcheze C, Sugantino M, Bittman R, Sacchettini JC. Crystal structure of the *Mycobacterium tuberculosis* enoyl-ACP reductase, InhA, in complex with NAD+ and a C16 fatty acyl substrate. J Biol Chem 1999; 274: 15582-89.

[62] Kuo MR, Morbidoni HR, Alland D, Sneddon SF, Gourlie BB, Staveski MM, *et al*. Targeting tuberculosis and malaria through inhibition of enoyl reductase. J Biol Chem 2003; 278: 20851-59.

[63] Roujeinikova A, Sedelnikova SE, de Boer GJ, Stuitje AR, Slabas AR, Rafferty JB, *et al*. Inhibitor binding studies on enoyl reductase reveal conformational changes related to substrate recognition. J Biol Chem 1999; 274: 30811-17.

[64] Baldock C, Rafferty JB, Sedelnikova SE, Baker PJ, Stuitje AR, Slabas AR, *et al*. A mechanism of drug action revealed by structural studies of enoyl reductase. Science 1996; 274: 2107-10.

[65] Perozzo R, Kuo M, Sidhu AS, Valiyaveettil JT, Bittman R, Jacobs WR Jr, *et al*. Structural elucidation of the specificity of the antibacterial agent triclosan for malarial enoyl acyl carrier protein reductase. J Biol Chem 2002; 277: 13106-114.

[66] McLeod R, Muench SP, Rafferty JB, Kyle DE, Mui EJ, Kirisits MJ, *et al*. Triclosan inhibits the growth of plasmodium falciparum and *Toxoplasma gondii* by inhibition of apicomplexan Fab I. Int J Parasitol 2001; 31: 109-13.

[67] Grassberger MA, Turnowsky F, Hildebrandt J. Preparation and antibacterial activities of new 1, 2, 3-diazaborine derivatives and analogues. J Med Chem 1984; 27: 947-53.

[68] Quemard A, Laneelle G, Lacave C. Mycolic acid biosynthesis: a target for ehtionamide in mycobacteria. Antimicrob Agents Chemother 1992; 36: 1316-21.

[69] Winder FG. In: Ratledge C, Stanford J Eds, The Biology of the Mycobacteria. Orlando, FA, Academic Press. 1982; 353-438.

[70] Stevens GJ, Hitchcock K, Wang YK, Coppola GM, Versace RW, Chin JA, *et al*. *In vitro* metabolism of N-(5-chloro-2-methylphenyl)-N'-(2-methylpropyl)thiourea: species comparison and identification od a novel thiocarbamide-glutathione adduct. Chem Res Toxicol 1997; 10: 733-41.

[71] de Barber AE, Mdluli K, Bosman M, Bekker LG, Barry CE. Ethionamide activation and sensitivity in multidrug-resistant *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 2000; 97: 9677-82.

[72] Nelson DR. Cytochrome P450 and the individuality of species. Arch Biochem Biophys 1999; 369: 1-10.

[73] Phetsuksiri B, Baulard AR, Cooper AM, Minnikin DE, Douglas JD, Besra GS, *et al*. Antimycobacterial activities of isoxyl and new derivatives through inhibition of mycolic acid biosynthesis. Antimicrob Agents Chemother 1999; 43: 1042-51.

[74] Nunn P, Porter J, Whitstanley P. Thiacetazone--avoid like poison or use with care? Trans R Soc Trop Med Hyg 1993; 87: 578-82.

[75] Decker CJ, Doerge DR. Covalent binding of 14C- and 35S-labeled thiocarbamides in rat hepatic microsomes. Pharmacol 1992; 43: 881-88.

[76] Hunter AL, Neal RA. Inhibition of hepatic mixed-function oxidase activity *in vitro* and *in vivo* by various thiono-sulfur-containing compounds. Biochem Pharmacol 1975; 24: 2199-205.

[77] Tan TH, The KS, Yang SY, Tan LS, Ko HL, Tjie HB, *et al*. Studies on cross-resistance between isoniazid, isoxyl, teebazone, ethionamide and ethambutol of tubercle bacilli from patients never treated with those drugs except with isoniazid. Paediatr Indones 1966; 6: 32-8.

[78] Osato T, Tsukagoshi K, Shimizu H. Studies on thiacetazone resistance of tubercle bacilli. 3. Cross resistance between thiacetazone and ethionamide. Kekkaku 1971; 46: 89-92.

[79] Pavelka MS, Jacobs WR Jr. Biosynthesis of diaminopimelate, the precursor of lysine and a component of peptidoglycan, is an essential function of *Mycobacterium smegmatis*. J Bacteriol 1996; 178: 6496-507.

[80] Almrud JJ, Oliveira MA, Kern AD, Grishin NV, Phillips MA, Hackert ML. Crystal structure of human ornithine decarboxylase at

2.1 A resolution: structural insights to antizyme binding. J Mol Biol 2000; 295: 7-16.

[81] Jackson LK, Goldsmith EJ, Phillips MA. X-ray structure determination of *Trypanosoma brucei* ornithine decarbxylase bound to D-ornithine and to G418: insights into substrate binding and ODC conformational flexibility. J Biol Chem 2003; 278: 22037-43.

[82] Grishin NV, Osterman AL, Brooks HB, Phillips MA, Goldsmith EJ. X-ray structure of ornithine decarboxylase from *Trypanosoma brucei*: The native structure and the structure in complex with a-difluoromethylornithine. Biochemistry 1999; 38: 15174-84.

[83] Bacchi CJ, Nathan HC, Hutner SH, McCann PP, Sjoerdsma A. Polyamine metabolism: a potential therapeutic target in trypanosomes. Science 1980; 210: 332-4.

[84] Gokulan K, Rupp B, Pavelka MS, Jacobs WR Jr, Sacchettini JC. Crystal structure of *Mycobacterium tuberculosis* diaminopimelate decarboxylase, an essential enzyme in bacterial lysine synthesis. J Biol Chem 2003; 278: 18588-96.

[85] Clements JM, Beckett RP, Brown A, Catlin G, Lobell M, Palan S, *et al*. Antibiotic activity and characterization of BB-3497, a novel peptide deformylase inhibitor. Antimicrob Agents Chemother 2001; 45: 563-70.

[86] Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC, *et al*. Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry 2000; 39: 1256-62.

[87] Serero A, Giglione C, Sardini A, Martinez-Sanz J, Meinnel T. An unusual peptide deformylase in the human mitochondrial N-terminal nethionine excision pathway. J Biol Chem 2003; 278: 52953-63.

[88] Giglione C, Serero A, Pierre M, Boisson B, Meinnel T. Indentification of eukaryotic peptide deformylases reveals universality of N-terminal protein processing mechanisms. EMBO J 2000; 19: 5916-29.

[89] Apfel C, Banner DW, Bur D, Dietz M, Hirata T, Hubschwerlen C, *et al*. Hydroxamic acid derivatives as potent deformylase inhibitors and antibacterial agents. J Med Chem 2000; 43: 2324-31.

[90] Becker A, Schlichting I, Kabsch W, Groche D, Schultz S, Wagner AFV. Iron center, substrate recognition and mechanism of peptide deformylase. Nat Struct Biol 1998; 5: 1053-58.

[91] Hao B, Gong W, Rajagopalan PT, Zhou Y, Pei D, Chan MK. Structural basis for the design of antibiotics targeting peptide deformylase. Biochemistry 1999; 38: 4712-9.

[92] Kumar A, Nguyen KT, Srivathsan S, Ornstein B, Turley S, Hirsh I, *et al*. Crystals of peptide deformylase from *Plasmodium falciparum* reveal critical characteristics of the active site for drug design. Structure 2002; 10: 357-67.

[93] Leung D, Abbenante G, Fairlie DP. Protease inhibitors: Current status and future prospects. J Med Chem 1999; 43: 305-41.

[94] Hackbarth CJ, Chen DZ, Lewis JG, Clark K, Mangold BL, Cramer JA, *et al*. N-alkyl urea hydroxamic acids as a new class of peptide deformylase inhibitors with antibacterial activity. Antimicrob Agents Chemother 2002; 46: 2752-64.

[95] Meinnel T, Patiny L, Ragusa S, Blanquet S. Design and synthesis of substrate analoge inhibitors of peptide deformylase. Biochemistry 1999; 38: 4287-95.

[96] Durand DJ, Green BG, O'Connell JF, Grant SK. Peptide aldehyde inhibitors of bacterial peptide deformylases. Arch Biochem Biophys 1999; 367: 297-302.

[97] Umezawa H, Aoyagi T, Tanaka T, Suda H, Okuyama A, Naganawa H, *et al*. Production of actinonin, an inhibitor of aminopeptidase M, by actinomycetes. J Antibiot (Toyko) 1985; 38: 1629-30.

[98] Giglione C, Vallon O, Meinnel T. Control of protein life-span by N-terminal methionine excision. EMBO J 2003; 22: 13-23.

[99] Pei D. Peptide deformylase: a target for novel antibiotics? Emerg Ther Targets 2001; 5: 23-40.

[100] Chan MK, Gong W, Rajagopalan PTR, Hao B, Tsai CM, Pei D. Crystal structure of *Escherichia coli* peptide deformylase. Biochemistry 1997; 36: 13904-09.

[101] Browner MF, Smith WW, Castelhano AL. Matrilysin-inhibitor complexes: common themes among metalloproteases. Biochemistry 1995; 34: 6602-10.

[102] Hu YJ, Wei Y, Zhou Y, Rajagopalan PT., Pei D. Determination of substrate specificity for peptide deformylase through the screening of a combinatorial peptide library. Biochemistry 1999; 38: 643-50.

[103] Hu X, Nguyen KT, Verlinde CL, Hol WG, Pei D. Structure-based design of a macrocyclic inhibitor of peptide deformylase. J Med Chem 2003; 46: 3771-4.

[104] Rajagopalan PT, Pei D. Oxygen-mediated inactivation of peptide deformylase. J Biol Chem 1998; 273: 22305-10.

[105] Rajagopalan PT, Grimme S, Pei D. Characterization of cobalt(II)-substituted peptide deformylase: function of the metal ion and the catalytic residue Glu-133. Biochemistry 2000; 39: 779-90.

[106] Yuan Z, Trias J, White RJ. Deformylase as a novel antibacterial target. Drug Discov Today 2001; 6: 954-61.

[107] Nguyen KT, Hu X, Colton C, Chakrabarti R, Zhu MX, Pei D. Characterization of human peptide deformylase: implications for antibacterial drug design. Biochemistry 2003; 42: 9952-8.

[108] Margolis PS, Hackbarth CJ, Young DC, Wang W, Chen DZ, Yuan Z, *et al*. Peptide deformylase in *Staphylococcus aureus*: resistance to inhibition is mediated by mutations in the formyltransferase gene. Antimicrob Agents Chemother 2000; 44: 1825-32.

[109] Apfel C, Locher H, Evers S, Takacs B, Hubschwerlen C, Pirson W, *et al*. Peptide deformylase as an antibacterial drug target: target validation and resistance development. Antimicrob Agents Chemother 2001; 45: 1058-64.

[110] Newton DT, Creuzenet C, Mangroo D. Formylation is not essential for initiation of protein synthesis in all eubacteria. J Biol Chem 1999; 274: 22143-6.

[111] Mazel D, Pochet S, Marliere P. Genetic characterization of polypeptide deformylase, a distinctive enzyme of eubacterial translation. EMBO J 1994; 13: 914-23.

[112] Elliott SD, Tai JY. The type-specific polysaccharides of *Streptococcus suis*. J Exp Med 1978; 148: 1699-704.

[113] McNeil M, Daffe M, Brennan PJ. Evidence for the nature of the link between the arabinogalactan and peptidoglycan components of the mycobacterial cell walls. J Biol Chem 1990; 265: 18200-6.

[114] Deng L, Mikusova K, Robuck KG, Scherman M, Brennan PJ, McNeil MR. Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. Antimicrob Agents Chemother 1995; 39: 694-701.

[115] Graninger M, Nidetzky B, Heinrichs DE, Whitfield C, Messner PJ. Chatacterization of the DTDP-4-dehydrorhamnose 3, 5-epimerase and dTDP-4pdehydrorhamnose reductase, required for dTDP-L-rhamnose biosynthesis in *Salmonella enterica* serovar Typhimurium LT2. J Biol Chem 1999; 274: 25069-77.

[116] Ma Y, Mills JA, Belisle JT, Vissa V, Howell M, Bowlin K, *et al*. Determination of the pathway for rhamnose biosynthesis in mycobacteria: cloning, sequencing and expression of the *Mycobacterium tuberculosis* gene encoding a-D-glucode-1-phosphate thymidylyltransferase. Microbiology 1997; 143: 937-45.

[117] Blankenfeldt W, Asuncion M, Lam JS, Naismith JH. The structural basis of the catalytic mechanism and regulation of glucose-1-phosphate thymidylyltransferase (RmlA). EMBO J 2000; 19: 6652-63.

[118] Allard ST, Giraud MF, Whitfield C, Graninger M, Messner PJ, Naismith JH. The crystal structure of dTDP-D-Glucose 4.6-dehydratase (RmlB) from *Salmonella enterica* serovar Typhimurium, the second enzyme in the dTDP-L-rhamnose pathway. J Mol Biol 2001; 307: 283-95.

[119] Allard ST, Beis K, Giraud MF, Hegeman AD, Gross JW, Wilmouth RC, *et al*. Towards a structural understanding of the dehydratase mechanism. Structure 2002; 10: 81-92.

[120] Christendat D, Saridakis V, Dharamsi A, Bochkarev A, Pai E, Arrowsmith CH, *et al*. Crystal structure of dTDP-4-keto-6-deoxy-D0-hexulose 3, 5-epimerase from *Methanobacterium thermoautotrophicum* complexed with dTDP. J Biol Chem 2000; 275: 24608-12.

[121] Giraud MF, Leonard GA, Field RA, Berlind C, Naismith JH. RmlC, the third enzyme of dTDP-L-rhamnose pathway, is a new class of epimerase. Nat Struct Biol 2000; 7: 398-402.

[122] Dong C, Major LL, Allen AG, Blankenfeldt W, Maskell DJ, Naismith JH. High-resolution structures of RmlC from *Streptococcus suis* in complex with substrate analogs locate the active site of thie class of enzyme. Structure 2003; 11: 715-23.

[123] Blankenfeldt W, Kerr ID, Giraud MF, McMiken HJ, Leonard GA, Whitfield C, *et al*. Variation on a theme of SDR. dTDP-6-deoxy-L-lyxo-4-hexulose reductase (RmlD) shows a new Mg2+ dependent dimerization mode. Structure 2002; 10: 773-86.

[124] Babaoglu K, Page MA, Jones VC, McNeil MR, Dong C, Naismith JH, *et al*. Novel inhibitors of an emerging target in *Mycobacterium tuberculosis*; Substituted thiazolidinones as inhibitors of dTDP-rhamnose synthesis. Bioorg Med Chem Lett 2003; 13: 3227-30.

[125] Ma Y, Stern RJ, Scherman MS, Vissa VD, Yan W, Jones VC, *et al*. Drug targetting *Mycobacterium tuberculosis* cell wall biosynthesis: genetics of dTDP-rhamnose synthetic enzymes and development of a microtiter plate-based screen for inhibitors of conversion of dTDP-glucose to dTDP-rhamnose. Antimicrob Agents Chemother 2001; 45: 1407-16.

[126] Pan F, Jackson M, Ma Y, McNeil M. Determination that the cell wall galactofuran synthesis is essential for growth of mycobacteria. J Bacteriol 2001; 178: 1047-52.

[127] Takayama K, Kilburn JO. Inhibition of synthesis of arabinogalactan by ethambutaol in *Mycobacterium smegmatis*. Antimicrob Agents Chemother 1989; 33: 1493-9.

[128] Belanger AE, Besra GS, Ford ME, Mikusova K, Belisle JT, Brennan PJ, *et al*. The embAB genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci USA 1996; 93: 11919-24.

[129] Holm L, Sander C. Protein structure comparison by alignment of distance matrices. J Mol Biol 1983; 233: 123-38.

[130] Sanders DA, Staines AG, McMahon SA, McNeil MR, Whitfield C, Naismith JH. UDP-galactopyranose mutase has a novel structure and mechanism. Nat Struct Biol 2001; 8: 858-63.

[131] Fullerton SW, Daff S, Sanders DA, Ingledew WJ, Whitfield C, Chapman SK, *et al*. Potentiometric analysis of UDP-galatopyranose mutase: Stablization of the flavosemiquinone by substrate. Biochemistry 2003; 42: 2104-9.

[132] Barlow JN, Girvin ME, Blanchard JS. Positional isotope exchange catalyzed by UDP-galactopyranose mutase. J Am Chem Soc 1999; 121: 6968-9.

[133] Togo H, He W, Waki Y, Yokoyama M. C-glycosylation technology with free radical reactions. Synlett 1998; 7: 700-17.

[134] Soltero-Higgin M, Carlson EE, Gruber TD, Kiessling LL. A unique catalytic mechanism for UDP-galactopyranose mutase. Nat Struct Mol Biol 2004; 11: 539-543.

[135] Scherman MS, Winans KA, Stern RJ, Jones VC, Bertozzi CR, McNeil MR. Drug targeting *Mycobacterium tuberculosis* cell wall synthesis: development of a microtiter plate-based screen for UDp-galactospyranose mutase and identification of an inhibitor from a uridine based library. Antimicrob Agents Chemother 2003; 47: 378-82.

[136] McKinney JD, Hoener zu Bentrup K, Munos-Elias EJ, Miczak A, Chen B, Chan W-T, *et al*. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxalate shunt enzyme isocitrate lyase. Nature 2000; 406: 735-8.

[137] Sharma V, Sharma S, Hoener zu Bentrup K, McKinney JD, Russell DG, Jacobs WR Jr, *et al*. Structure of isocitrate lyase, a persistence factor of *Mycobacterium tuberculosis*. Nat Struct Biol 2000; 7: 663-8.

[138] Britton KL, Abersinghe IS, Baker PJ, Barynin V, Diehl P, Langridge SJ. The structure and domain organization of *Escherichia coli* isocitrate lyase. Acta Crystallogr 2001; 57: 1209-18.

[139] Martinussen J, Schallert J, Andersen B, Hammer K. The pyrimidine operon pyrRPB-car-A from *Lactococcus lactis*. J Bacteriol 2001; 183: 2785-94.

[140] Nyka W. Studies on the effects of starvation on mycobacteria. Infect Immun 1974; 9: 843-50.

[141] Rodriguez G, Voskuil MI, Gold B, Schoolnik K, Smith I. IdeR, an essential gene in *Mycobacterium tuberculosis*: Role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. Infect Immun 2002; 70: 3371-81.

[142] Chan K, Knaak T, Satkamp L, Humbert O, Falkow S, Ramakrishnan L. Complex pattern of *Mycobacterium marinum* gene expression during long-term granulomatous infection. Proc Natl Acad Sci USA 2002; 99: 3920-25.

[143] Tomchick DR, Turner RJ, Switzer RL, Smith JL. Adaptation of an enzyme to regulatory function: structure of Bacillus subtilis PyrR, a pyr RNA-binding attentuation protein and uracil phosphori-bosyltransferase. Structure 1998; 6: 337-50.

[144] Kadziola A, Neuhard J, Larsen S. Structure of product-bound Bacillus caldolyticus uracil phosphoribosyltransferase confirms ordered sequential substrate binding. Acta Crystallogr 2002; 58: 936-45.

[145] Schumacher MA, Carter D, Scott DM, Roos DS, Ullman B, Brennan RG. Crystal structures of *Toxoplasma gondii* phosphoribosyltransferase reveal the atomic basis of pyrimidine discrimination and prodrug binding. EMBO J 1998; 17: 3219-32.

[146] Blakely RL. Eukaryotic dihydrofolate reductase. Adv Enzymol Relat Areas Mol Biol 1995; 70: 23-102.

[147] Blakely RL. In: Blakely RL, Benkovic SJ Eds, Folates and Pterins. New York, Wiley, 1984; 191-253.

[148] Schweitzer MR, Dicker AP, Bertino JR. Dihydrofolate reductase as a therapeutic target. FASEB J 1990; 4: 2441-52.

[149] Pitts C, Bowen D, Southerland WM. Interaction energy analyses of folate analog binding to human dihydrofolate reductase: contribution of the antifolate substructural regions to complex stability. Drug Metabol Drug Interact 2000; 16: 99-121.

[150] Cody V, Galitsky N, Luft JR, Pangborn W, Rosowsky A, Queener SF. Structure-based enzyme inhibitor design: modeling studies and crystal structure analysis of *Pheumocystis carinii* dihydrofolate reductase ternary complex with PT653 and NADPH. Acta Crystallogr 2002; 58: 946-54.

[151] Rongbao L, Sirawaraporn R, Chitnumsub P, Sirawaraporn W, Wooden J, Athappilly F, *et al*. Three-dimensional structure of *M. tuberculosis* dihydrofolate reductase reveals opportunities for the design of novel tuberculosis drugs. J Mol Biol 2000; 295: 307-23.

[152] Matthews DA, Alden RA, Bolin JT, Freer ST, Hamlin R, Xuong N, *et al*. Dihydrofolate reductase: X-ray structure of the binary complex with methotrexate. Science 1977; 197: 452-5.

[153] Coates A, Hu Y, Bax R, Page C. The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov 2002; 1: 895-910.

[154] Michison DA. Mechanisms of tuberculosis chemotherapy. J Pharm Pharmacol 1997; 49: 31-6.

[155] Schumann G, Mollmann U. Screening system for xenosiderophores as potential drug delivery agents in mycobacteria. Antimicrob Agents Chemother 2001; 45: 1317-22.

[156] Abagyan R, Batalov S, Cardozo T, Totrov M, Webber J, Zhou Y. Homology modeling with internal coordinate mechanics: deformation zone mapping and improvements of models *via* conformational search. Proteins 1997; Suppl 11: 29-37.

[157] Kantardjieff KA, Kim C-Y, Naranjo C, Waldo GS, Lekin T, Segelke BW, *et al*. *Mycobacterium tuberculosis* RmlC epimerase (Rv3465): a promising drug-target structure in the rhamnose pathway. Acta Crystallog 2004; D60: 895-902.

[158] Haney SA, Alksne LE, Dunman PM, Murphy E, Projan SJ. Genomics in anti-infective drug discovery--getting to endgame. Curr Pharm Design 2002; 8(13): 1099-118.

[159] Nagaraja V, Sikder D, Jain P. DNA topoisomerase I from mycobacteria--a potential drug target. Curr Pharm Design 2002; 8(22): 1995-2007.

[160] Teodoro ML, Kavraki LE. Conformational flexibility models for the receptor in structure based drug design. Curr Pharm Design 2003; 9(20): 1635-48.